Role of micronutrients during fungal infections

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1. Summary

Trace metals serve as structural and catalytic cofactors for numerous proteins and thus are crucial for all living organisms. Iron is the fourth most abundant element in the Earth's crust and the most abundant metal in humans. The presence of redox active iron in living systems is critical for oxygen transport, energy production, DNA repair and replication, gene expression, and transcription. Zinc, the twenty fifth most abundant element in the Earth's crust, is the second most abundant transition metal in vertebrates. Zinc is a redox inactive trace metal and its availability in organisms is indispensable for the function of more than 300 enzymes, DNA stabilization and synthesis, gene expression, protein synthesis, and immunity.

Essential trace metals are required at various concentrations depending on the organism and the cellular function. The disturbance of optimal metals level is extremely harmful for cells, thus metal concentrations are tightly controlled within organisms. Correspondingly, the availability of metals is recognized as the central factor in infections.

The host is able to both limit and overload iron and zinc levels in order to inhibit microbial growth in a process known as nutritional immunity. Depending on the pathogen species and their location within the organism, the host regulates metal levels from extreme deficiency in the blood to overload in phagosomes. In return, pathogens have evolved to counteract host mediated metal fluctuations. Under metal starvation pathogens rely on plasma membrane metal importers, chelators production, and host metalloproteins utilization. In response to metal toxicity pathogenic microorganisms make the use of plasma membrane metals exporters and intracellular detoxification systems.

Candida albicans is a fungus that co-exists as a harmless commensal in humans, however, when the immune system is compromised or host's barriers are damaged, *C. albicans* is able to cause superficial to severe systemic infections. During commensalism and infection, the fungus successfully inhabits host niches that dramatically vary in metals availability. Thus *C. albicans* is able to precisely regulate metals homeostasis *via* the comprehensive transcriptional network that controls metals assimilation, storage, detoxification, and mobilization processes.

The current work is focused on iron and zinc homeostasis and its role in survival and virulence of *C. albicans*. The major finding of this thesis is that *C. albicans* has evolved to cope with metals fluctuations and some of its strategies are unique in *C. albicans*, having not been described for other fungi. The regulation of iron homeostasis, in addition to other factors, is regulated *via* Hap43, a CCAAT-binding

transcription factor. Hap43 has a high degree of overlap in sequence, domains structure, and gene regulation under iron limitation with its orthologue HapX in a distantly related pathogenic fungus *Aspergillus fumigatus*. However, in contrast to HapX, Hap43 is not required for growth under iron excess in *C. albicans*. Furthermore, we characterized the transcriptional response to zinc limitation and found processes such as transcription, translation, metabolism, and biosynthesis to be affected by zinc starvation. Under low zinc levels *C. albicans* relies on pH-dependent plasma membrane zinc importers and such a system is analogous to *A. fumigatus*. Under zinc excess levels *C. albicans* rapidly accumulates zinc into zincosomes, unlike *Saccharomyces cerevisiae* that immediately compartmentalizes zinc into vacuoles. Additionally, zinc uptake, storage, and detoxification are critical for *C. albicans* virulence. These findings demonstrate the metal homeostasis regulation to be essential for survival of *C. albicans* within the host. Furthermore, the previously available information and our data indicate that fungi have developed various strategies to cope with changes of metal concentrations in their environment.

2. Zusammenfassung

Spurenlemente dienen als strukturelle und katalytische Co-faktoren für viele Proteine und sind somit unabdingbar für alle Lebewesen. Eisen ist das vierthäufigste Element der Erdkruste und das am häufigsten vorkommende Metall im Menschen. Das Vorhandensein von redox-aktivem Eisen in lebenden Organismen ist entscheidend für Sauerstofftransport, Energieerzeugung, DNA-Reparatur und -replikation, Genexpression und Transkription. Zink, das 25häufigste Element der Erdkruste, ist das zweithäufigste Metall in Wirbeltieren. Zink ist ein redox-inaktives Spurenelement und dessen Verfügbarkeit im Organismus ist unerlässlich für die Funktion von mehr als 300 Enzymen, für DNA Stabilisierung und -synthese, Genexpression, Proteinsynthese und das Immunsystem.

Essentielle Spurenelemente werden entsprechend des Organismus und der zellulären Funktion in verschiedenen Konzentrationen benötigt. Eine Störung des Metallhaushaltes ist sehr schädlich für Zellen, weshalb die Konzentration an Metallen im Organismus streng reguliert wird. Die Verfügbarkeit an Metallen wird daher auch als ausschlaggebender Faktor während Infektionen angesehen.

Der Wirt ist in der Lage Eisen- und Zinklevel sowohl zu beschränken als auch überzudosieren um das Wachstum von Mikroorganismen zu hemmen; ein Prozess der als nährstoffbasierte Immunität („nutritional immunity“) bekannt ist. In Abhängigkeit des Krankheitserregers und dessen Lokalisation im Organismus können Metalle extrem limitiert sein (z.B. im Blut) oder andererseits äußerst hohe Konzentrationen erreichen (z.B. im Phagosom). Im Gegenzug haben Krankheitserreger gelernt, den wirtsvermittelten Metallschwankungen entgegenzuwirken. Bei einem Metallmangel verwenden Krankheitserreger in erhöhtem Maße membrangängige Metallimporter, Chelatoren oder Metalloproteine des Wirts. Bei einem Metallüberschuss benutzen Krankheitserreger verstärkt Metallexporter in der Plasmamembran oder intrazelluläre Entgiftungssysteme.

Candida albicans ist ein Hefepilz, der als harmloser Kommensale im Menschen existiert, jedoch unter Immunsuppression oder bei Schädigung der Wirtsbarrieren auch oberflächliche bis hin zu schweren systemischen Infektionen auslösen kann. Während Kommensalismus und Infektion kann der Pilz Wirtsnischen besiedeln, die sich in ihrer Verfügbarkeit an Metallen drastisch unterscheiden. Eine präzise Regulation des Metallgleichgewichts in *C. albicans* wird durch ein umfassendes Transkriptionsnetzwerk ermöglicht, welches die Metallaufnahme, -lagerung, detoxifizierung und -mobilisierung kontrolliert.

Zusammenfassung

Die vorliegende Arbeit konzentriert sich auf die Eisen- und Zinkhomöostase und deren Rolle im Überleben und der Virulenz von *C. albicans*. Das hauptsächliche Ergebnis dieser Arbeit ist, dass sich während der Evolution von *C. albicans* einzigartige Strategien entwickelt haben, um mit Metallschwankungen umzugehen, die bisher in keinem anderen Pilz beschrieben worden sind. Die Regulation des Eisenhaushalts wird unter anderem durch Hap43 reguliert, einem CCAAT-bindenden Transkriptionsfaktor. Hap43 hat große Ähnlichkeit mit dem orthologen Gen HapX aus dem entfernt verwandten pathogenem Pilz *Aspergillus fumigatus* bezüglich Sequenz, Domänenstruktur und Genregulation unter Eisenmangel. Jedoch ist Hap43 im Gegensatz zu HapX nicht für Wachstum unter Eisenüberschuss nötig. Antwort auf einen Zinkmangel charakterisiert, die viele Prozesse wie Transkription, Translation, Stoffwechsel und Biosynthesen beeinflusst. Bei geringen Zinkkonzentrationen verlässt sich *C. albicans*, analog zu *A. fumigatus*, auf pH-abhängige Plasmamembran-Zinkimporter. Während eines Zinküberschusses sammelt *C. albicans* Zink in speziellen Zinkosomen, während *Saccharomyces cerevisiae* beispielsweise Zink sofort in Vakuolen speichert. Weiterhin sind Zinkaufnahme, -lagerung und -entgiftung entscheidend für die Virulenz von *C. albicans*. Diese Ergebnisse zeigen, dass die Metallhomöostase für das Überleben von *C. albicans* im Wirt essentiell ist. Vorangegangene Studien sowie die Daten dieser Arbeit weisen darauf hin, dass Pilze verschiedene Strategien entwickelt haben, um mit Schwankungen an Metallkonzentrationen in ihrer Umwelt zurechtzukommen.

3. Introduction

3.1. Metals in biology

Trace metals are crucial for all forms of life, as they are structural and catalytic cofactors in a multitude of biological processes, including, but not limited to, DNA replication, transcription, respiration, metabolic pathways, and responses to oxidative stress. Although the transition metals, which include vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) are micronutrients that are required in minute quantities, they are involved in critical physiological pathways (Goldhaber 2003, Haraguchia 2004). Due to their unique properties, transition metals are often incorporated in metalloenzymes, storage and transport proteins, and transcription factors (TFs). In fact, it has been bioinformatically estimated that 50% of all enzymes require metal ions for their function (Andreini, Bertini et al. 2009).

However, an excess of metals can be toxic to cells. The first-row trace metals, except for zinc, are redox active, due to their unfilled d-orbitals, and thus the accumulation of metals can lead to the production of hydroxyl and hydroperoxyl radicals *via* the Fenton reaction (Halliwell and Gutteridge 1984). These free radicals have an adverse effect on nucleic acids, lipids, and proteins by altering their redox status and increasing the level of oxidative stress (Phaniendra, Jestadi et al. 2015). Additionally, when present in high concentrations, these metals compete with other cognate metals for metal-binding sites in enzymes (McDevitt, Ogunniyi et al. 2011, Gu and Imlay 2013). According to the Irving-Williams Series, the relative stabilities of transition metal complexes increase across the period to a maximum stability at nickel (Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)) (Irving and Williams 1948), although, as described by the Jahn-Teller postulates, the orbital degeneracy is eliminated by distortion in copper octahedral compounds, making copper complexes more stable than nickel complexes (Jahn 1937).Thus, even a slight increase above the optimal metal concentration can lead to the replacement of metals like manganese or iron from their metalloprotein binding sites, causing a loss of the proteins' function (Jahn 1937, Irving and Williams 1948).

Consequently, as both limitation and excess of transition metals are harmful to cells, the levels of metals must be carefully controlled in all living organisms.

Nutritional immunity

Given their crucial role in biological processes, the availability of trace metals is considered to be a central factor during infections: The host restricts access to trace metals in order to inhibit microbial growth in a process known as “nutritional immunity” (Weinberg 1975), while pathogens apply various strategies to obtain metals from the host (Hood and Skaar 2012).

Nutritional immunity has already been described for manganese, iron, cobalt, nickel, copper, and zinc. The current state of knowledge on the battlefield for these metals during bacterial infections is summarized in (Palmer and Skaar 2016), during fungal infections in (Gerwien, Skrahina et al. 2018), and during viral infections in (Drakesmith and Prentice 2008, Lazarczyk and Favre 2008). Here, the concept of nutritional immunity and microbial metal homeostasis will be described for zinc and iron, as these metals are the focus of this thesis.

Iron in the host defense

Among all of the known transition metals, iron is considered to be vital for all living organisms, and even viruses (Drakesmith and Prentice 2008). So far, only a single bacterium, *Borrelia burgdorferi*, the etiologic agent of Lyme disease, is known to be able to exist without iron. In a unique evolutionary development, manganese is used as a cofactor in *B. burgdorferi* instead of iron (Posey and Gherardini 2000). The human body requires iron for oxygen storage, transport, and sensing (hemoglobin, myoglobin, and hypoxia-inducible factor prolyl hydroxylases) (Hurrell 1997, Bianchi, Tacchini et al. 1999, McDowell 2003, Peyssonnaux, Zinkernagel et al. 2007); energy production (cytochrome c oxidase and NADH dehydrogenase) (Schejter and Plotkin 1988); metabolism and detoxification pathways (amino acid oxidases, fatty acid desaturases, and cytochrome P450) (Crandall 1955, Schejter and Plotkin 1988, Hanukoglu 2006); and iron storage and transport (transferrin and ferritin)(Crichton and Charloteaux-Wauters 1987, Casiday 2000).

Although iron is one of the most abundant elements in the Earth’s crust, its oxidized ferric state has a low solubility. *In vivo* at physiological oxygen levels and neutral pH, iron is readily oxidized from the soluble ferrous (Fe^{2+}) to the insoluble ferric (Fe^{3+}) state, which has an extremely low bioavailability (Klausner and Rouault 1996, Ilbert and Bonnefoy 2013). Iron is able to both donate and accept electrons and thus, the reversible changes between the ferrous and ferric iron redox states, allows iron to catalyze electron-transfer reactions in cells. At elevated concentrations iron is toxic due to the ability of iron to convert hydrogen peroxide into free radicals (Andrews 2000).

Therefore, the concentration of free iron ions is extremely low in cells. However, some iron ions are bound to low-affinity molecules and in such state iron is accessible (called the labile or "free" iron pool) and provides the necessary iron for immediate use by the cell. In mammals, the intracellular labile iron concentration is less than 1 micro molar, or about 5% of the total cellular iron content (Kakhlon and Cabantchik 2002). The remaining iron is incorporated as a heme compound into proteins (further called hemoproteins): hemoglobin, myoglobin, and heme enzymes (catalases, peroxidases, nitric oxide synthase, and cytochromes) (Alderton, Cooper et al. 2001, Paoli, Marles-Wright et al. 2002). Additionally, iron is incorporated in enzymes and proteins as a non-heme compound in the form of flavin-iron enzymes, transferrin, and finally ferritin, the major intracellular storage molecule for iron (McDowell 2003).

The host applies several layers of regulation, including systemic and local, to affect the iron levels faced by microbes.

Systemic control of iron levels is mediated by the peptide hormone hepcidin (Nicolas, Chauvet et al. 2002). During infections hepcidin levels increase, which leads to the reduction of the iron concentration in plasma by an inhibition of iron absorption from the gut and an increase of iron sequestration by macrophages (Goodnough, Nemeth et al. 2010). This hepcidin-induced anemia has been described as an effective defense mechanism against microbial, protozoan, and viral infections (Nicolas, Chauvet et al. 2002, Lundgren and Mocroft 2003, Portugal, Carret et al. 2011). Interleukin-6 (IL-6) (Nemeth, Rivera et al. 2004), activin B (Besson-Fournier, Latour et al. 2012), type I interferons (IFNs) (Ryan, Altamura et al. 2012, Ichiki, Ikuta et al. 2014), and bone morphogenetic protein 2 (BMP2) (Maes, Nemeth et al. 2010) trigger hepcidin synthesis in the liver in response to infections. Additionally, hepcidin induces the production of ferritin, transferrin, lactoferrin, and haemoglobin, the proteins that bind iron ions with high affinity. For example, the concentration of ferritin and transferrin in serum (Custer, Finch et al. 1995, Gomme, McCann et al. 2005, Ong, Ho et al. 2006, Wu, Chen et al. 2014); lactoferrin in breast milk, tears, saliva, and leukocytes is increased during inflammation and infection (Schaible, Collins et al. 2002, Kane, Sandborn et al. 2003, Cassat and Skaar 2013). During cell lysis in the blood, hemoglobin binds haptoglobin, and heme - hemopexin and the abundance of both haptoglobin and haemopexin is induced by inflammatory cytokines (Schaer, Buehler et al. 2013, Graw, Mayeur et al. 2016). In addition to iron ion chelating proteins, an acute-phase neutrophil gelatinase-associated lipocalin-2 protein (also called siderocalin) chelates iron-loaded siderophores, produced by bacteria (Flo, Smith et al. 2004, Malyszko, Tesar et al. 2010), and thus this leads to the inhibition of bacterial growth (Nairz, Theurl et al. 2009).

Local host mediated iron limitation occurs on a cellular level. As an example, in response to an intracellular pathogen *Plasmodium falciparum,* the host reduces the level of ferritin in hepatocytes and hemoglobin in erythrocytes (Gwamaka, Kurtis et al. 2012, Clark, Goheen et al. 2014). Furthermore, the phagosome iron concentration is controlled *via* plasma membrane natural resistance-associated macrophage protein 1 (NRAMP1) that exports iron and manganese out of the phagosome. NRAMP1 levels are induced by lipopolysaccharide and IFNγ and its presence is essential to combat intracellular infections (Canonne-Hergaux, Gruenheid et al. 1999), including the infection by *Mycobacterium tuberculosis* (Li, Yang et al. 2011).

So far little is known regarding iron overload as a host response to infections. Recent studies have shown that the host is able to mediate iron overload (which leads to accumulation of reactive oxygen species (ROS) (Halliwell and Gutteridge 1984)) within phagosomes to inhibit the growth of Group A *streptococcus* (GAS) (VanderWal, Makthal et al. 2017). Furthermore, pathogens actively express high affinity iron uptake transporters in iron limited niches such as blood. The rapid switch to iron sufficient levels, for example in phagosomes (VanderWal, Makthal et al. 2017), might lead to microbial killing, due to insufficient or slow down-regulation of iron importers, and therefore, to iron over-accumulation within the pathogen (Gsaller, Hortschansky et al. 2014).

Zinc in the host defense

Zinc is the second most abundant trace metal in biological systems after iron (Wapnir 1990) and the only metal that can be found in all six functional classes of enzymes (Andreini, Banci et al. 2006, Sousa, Lopes et al. 2009). Life on Earth is believed to have started with photosynthesizing zinc sulfide compartments, and thus zinc is considered to be a key element in the origin of life (Mulkidjanian and Galperin 2009). However, like iron, zinc has a low accessibility to microorganisms in host niches, as it is barely soluble at neutral pH levels.

In the early immune response to infections, the host is able to decrease serum zinc levels systemically by up to 80% (called hypozincemia) (Gaetke, McClain et al. 1997, Liuzzi, Lichten et al. 2005) mainly *via* the induced expression of *ZIP14*, a hepatocyte plasma membrane zinc transporter, and the induced production of metallothioneins (MTs) in the liver and kidneys (Cousins and Leinart 1988, Zangger, Oz et al. 2001, Cousins, Liuzzi et al. 2006, Subramanian Vignesh and Deepe 2017). In addition, the host increases the levels of zinc binding proteins in response to infections. Histatins, antimicrobial peptides that are mainly present in the oral cavity, chelate zinc as well as copper and restrict fungal growth (Gusman, Lendenmann et al. 2001). The

S100 family of vertebrates Ca binding proteins includes: S100A7 (psoriasin), which exhibits antimicrobial properties against *Escherichia coli* and dermatophytes by zinc chelation at mucosal surfaces (Glaser, Harder et al. 2005, Fritz, Beck-Jendroschek et al. 2012); and S100A12 (calgranulin C), which both sequesters zinc and copper away from pathogens and induces oxidative stress by generating superoxides (Moroz, Antson et al. 2003, Moroz, Burkitt et al. 2009). Interestingly, pathogens require metal containing superoxide dismutases (SODs) to combat ROS generated by the host. Thus calgranulin C efficiency is high due to its parallel metal chelation and ROS production functions. The host protein calprotectin is able to chelate zinc, manganese, iron, nickel, and copper (Nakashige, Zhang et al. 2015, Kelliher and Kehl-Fie 2016, Baker, Nakashige et al. 2017, Besold, Gilston et al. 2017, Nakashige, Zygiel et al. 2017). Calprotectin is a heterodimer, composed of two subunits: S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14), and accounts for 45% of the neutrophil cytoplasmic protein content (Edgeworth, Gorman et al. 1991). By virtue of metals chelation, calprotectin possesses a broad antimicrobial activity against bacterial and fungal infections (Urban, Ermert et al. 2009). Importantly, it is present in neutrophil extracellular traps (NETs), which are released by neutrophils upon stimulation with pathogens, and consist of DNA associated with histones, lactoferrin, elastase, myeloperoxidase, and calprotectin (Urban, Ermert et al. 2009). Surprisingly, calprotectin's zinc chelation property has been found to comprise the main mechanism against fungal pathogens, as both treatments of NETs with anti-calprotectin antibodies or zinc supplementation reduces the NETs' antimicrobial activity against fungi (Urban, Ermert et al. 2009).

In addition to the action of extracellular zinc binding proteins, the host is able to change zinc levels inside its cells to create a zinc limited environment for intracellular microorganisms. Activated phagocytes decrease their lysosomal zinc content *via* the expression of the zinc exporter *ZIP8* (Begum, Kobayashi et al. 2002, Kitamura, Morikawa et al. 2006, Aydemir, Liuzzi et al. 2009). Additionally, activated macrophages move zinc out of the cytoplasm into the Golgi apparatus and also sequester zinc away into MTs (Vignesh, Figueroa et al. 2013).

As with iron, the host can apply zinc's toxic properties and accumulate zinc within certain niches in order to combat pathogens. For example, an increase of intraphagosomal zinc levels was shown to mediate resistance against *M. tuberculosis* and GAS (Botella, Peyron et al. 2011, Ong, Gillen et al. 2014).

Pathogens response to metal fluctuations

Viruses, protozoa, bacteria, and fungi that infect vertebrates have evolved a plethora of different strategies to counteract the host defense mechanisms.

Viruses are an interesting example, as their whole replication cycle is metal-dependent (Chaturvedi and Shrivastava 2005). Recently, there have been reports of viruses actively modulating cellular metal metabolism in order to establish an optimal replication environment. Human cytomegalovirus (HCMV), for example, possesses a protein US2 that binds the host's hemochromatosis-associated protein (HFE), which is bound to transferrin receptor 1 (TfR1) in uninfected cells. This US2–HFE interaction leads to an accumulation of iron in HCMV-infected cells, which promotes viral growth (Ben-Arieh, Zimerman et al. 2001, Vahdati-Ben Arieh, Laham et al. 2003). A similar mechanism was described for HIV-1 Nef protein, where a Nef-HFE complex leads to increased iron levels in HIV-1-infected cells (Drakesmith, Chen et al. 2005). Additionally, the hepatitis C virus is able to upregulate TfR1 in hepatic cells and thus increases an intracellular iron content (Saito, Fujimoto et al. 2005). A viral strategy for the acquisition of zinc in host cells was described for human papillomavirus (HPV), whose E5 protein interacts with host ZnT1 protein, a plasma membrane zinc transporter, to increase the intracellular zinc level, which benefits viral replication (Lazarczyk, Pons et al. 2008).

Pathogenic protozoans are also able to counteract host-mediated nutritional immunity, and this is certainly best investigated for *Plasmodium* species. During blood-stage infection *P. falciparum* is able to use ferritin as an iron source (Coronado, Nadovich et al. 2014). Zinc inside the erythrocytes is required for *P. falciparum* growth and development and the predicted zinc importer (PF07_0065) was found to be upregulated during the blood stage infection (Marvin, Wolford et al. 2012). The iron and zinc importer in *P. berghei*, ZIPCO, was described to be essential for its development inside hepatocytes (Sahu, Boisson et al. 2014). *Leishmania amazonensis,* an intracellular parasite*,* is also able to manipulate intracellular macrophage iron levels *via* the inhibition of the host iron exporter ferroportin expression in macrophages (Ben-Othman, Flannery et al. 2014).

Among all pathogens, the bacterial strategies on counteracting host-mediated metal limitation and overload are best studied. The mechanisms of subverting the host mediated metal limitation include high-affinity metal importers, siderophores uptake, and the utilization of host metalloproteins. Gram-negative bacteria transport ferric iron from the extracellular space *via* an outer membrane receptor, a periplasmic binding protein (PBP), and an inner-membrane ATP-binding cassette (ABC) transporter. Gram-positive bacteria, lacking an outer membrane, rely only on the membrane-associated

ABC transport system (Clarke, Tari et al. 2001, Miethke and Marahiel 2007). Ferrous iron is transported by the FeoB family of cytoplasmic membrane transporters (Cartron, Maddocks et al. 2006, Aranda, Cortes et al. 2009). Siderophores, secreted by bacteria, bind ferric iron with the affinity that exceeds that of transferrin and lactoferrin (Schalk 2008). Bacteria that do not have genes required for siderophores production in their genomes are able to utilize siderophores produced by other bacteria (called xenosiderophores). This phenomenon is called “siderophore cheating” and this strategy is beneficial to non-producers, as they gain a fitness advantage over producers (Butaite, Baumgartner et al. 2017). Heme, hemoglobin, transferrin, and lactoferrin are utilized by bacteria as an iron source. From all of these proteins, except for heme that is taken as a whole molecule, iron is extracted prior to the transport *via* membrane receptors, periplasmic binding proteins, and the ABC transporter system. To cope with the metal toxicity, bacteria encode metal membrane exporters, which allow them to prevent metal overload within phagosomes (Guan, Pinochet-Barros et al. 2015, Pi, Patel et al. 2016). These well described strategies are summarized in recently published reviews (Hood and Skaar 2012, Palmer and Skaar 2016).

The number of pathogenic fungi is relatively low, but these few pathogenic species frequently cause diseases, including severe infections, which lead to high mobility and mortality rates (Brown, Denning et al. 2012). Within the host, fungi also face metal fluctuations and thus have evolved a number of mechanisms to cope with nutritional immunity. However, some strategies radically differ from those of bacteria. The focus of the current thesis was to analyze the fungal metal homeostasis and the nutritional immunity concept in the host-fungal interactions. *Candida albicans* was used as a model fungus in order to investigate zinc and iron homeostasis.

3.2. Epidemiology of fungal infections

There are 611,000 described species of fungi and about 600 species are considered to be human pathogens. However, only few of these are responsible for the vast majority of all fungal infections (Mora, Tittensor et al. 2011, Brown, Denning et al. 2012). The impact of pathogenic fungi on humans health can be seen in current epidemiological data (**Table 1**) (Brown, Denning et al. 2012). The mortality rate of invasive fungal infections is above 50% in average even under antifungal therapy. Although 1.5 million people die each year from invasive fungal infections, which is almost as many as from tuberculosis (1.7 million deaths in 2016) (WHO 2016), the importance of fungal infections is continuously underestimated – and the World Health Organization (WHO) does not have a department dedicated to fungal diseases (Brown, Denning et al. 2012).

Table 1. Statistics of the 10 most significant invasive fungal infections, adapted from (Brown, Denning et al. 2012).

Disease (most common species)	Location	Estimated life-threatening infections/ year at that location	Mortality rates (% in infected populations)
Opportunistic invasive mycoses			
Aspergillosis (*Aspergillus fumigatus*)	Worldwide	> 200,000	30 – 95
Candidiasis (*Candida albicans*)	Worldwide	> 400,000	46 – 75
Cryptococcosis (*Cryptococcus neoformans*)	Worldwide	> 1,000,000	20 – 70
Mucormycosis (*Rhizopus oryzae*)	Worldwide	> 10,000	30 – 90
Pneumocystis (*Pneumocystis jirovecii*)	Worldwide	> 400,000	20 – 80
Endemic dimorphic mycoses			
Blastomycosis (*Blastomyces dermatitidis*)	Midwestern Atlantic United States	≈ 3,000	< 2 – 68
Coccidioidomycosis (*Coccidioides immitis*)	Southwestern United States	≈ 25,000	< 1 – 70
Histoplasmosis (*Histoplasma capsulatum*)	Midwestern United States	≈ 25,000	28 – 50
Paracoccidioidomycosis (*Paracoccidioides brasiliensis*)	Brazil	≈ 4,000	5 – 27
Penicilliosis (*Penicillium marneffei*)	Southeast Asia	> 8,000	2 – 75

The genus *Candida*

Although the genus *Candida* represents approximately 150 species, more than 90% of infections are caused by only five species: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (Samaranayake and Samaranayake 1994, Sardi, Scorzoni et al. 2013, Guinea 2014). *C. albicans, C. parapsilosis*, and *C. glabrata* are commensals of mucosal surfaces (Sardi, Scorzoni et al. 2013), *C. parapsilosis* is typically present on skin (Trofa, Gacser et al. 2008), and *C. tropicalis* and *C. krusei* are environmental fungi (Samaranayake and Samaranayake 1994).

Candidiasis

Candidiasis is a superficial or systemic infection caused by *Candida* species. *C. albicans* is the most common cause of both superficial and systemic candidiasis in humans (CDC 2013). Among superficial mucosal infections, the most common is vulvovaginal candidiasis, and *de facto* 5 to 8% women experience four or more proven incidents during their lifetime (Sobel 2007). Oral candidiasis is reported in 60% of HIV-infected patients and 80% in the case of AIDS (McCarthy, Mackie et al. 1991, Palmer, Robinson et al. 1996). Candidemia, the presence of *Candida* species in the blood, is the most common form of invasive candidiasis and the most frequent bloodstream

infection in the USA (Wisplinghoff, Bischoff et al. 2004, Magill, Edwards et al. 2014). There are 46,000 cases of invasive candidiasis reported each year in the USA (CDC 2013).

Candida albicans

C. albicans belongs to a CTG clade of asexual *Candida* species, whose defining feature is that the CTG codon is translated into serine instead of leucine (Santos and Tuite 1995). *C. albicans* is a diploid organism and its 16 Mbp haploid genome consists of eight chromosomes with an estimated 6,735 ORFs (Jones, Federspiel et al. 2004). A complete meiotic sexual cycle was not identified in *C. albicans*, although the existence of haploid forms and fusion events of two mating types were described, as well as a parasexual cycle (Hull, Raisner et al. 2000, Hickman, Zeng et al. 2013).

C. albicans is commonly found as a harmless commensal in the microbiome of the gastrointestinal tract and the oral cavity in healthy humans. However, it is also able to cause life-threatening systemic infections, especially in immunocompromised individuals or upon damage of the mucosal barriers. During infection *C. albicans* relies on a range of virulence factors and fitness attributes.

3.3. *Candida albicans* pathogenicity mechanisms

Polymorphism

C. albicans is an polymorphic fungus that can grow as budding yeasts; elongated conjoined yeasts that form pseudohyphae; parallel-sided true hyphae; can form chlamydospores (Barnes, Flesher et al. 1971); giant yeast cells (Goliath cells) (Malavia, Lehtovirta-Morley et al. 2017); and intestinal gut form cells (Pande, Chen et al. 2013). Further morphological changes include white-grey-opaque cell switching phenotypes (Slutsky, Staebell et al. 1987); elongated shmoo-mating forms, which lead to tetraploid zygote formation (Kachurina 2009); and trimera that are formed by unequal chromosome segregation (Harrison, Hashemi et al. 2014).

The yeast and hyphae forms are clinically relevant, and the ability of *C. albicans* to perform yeast-to-hyphae transitions is considered critical for its pathogenicity (Jacobsen, Wilson et al. 2012). The yeast form is proposed to be mainly involved in dissemination (Berman and Sudbery 2002), while the hyphae form is essential for invasion into the host tissue, causing its damage (Sudbery 2011, Tyc, Kuhn et al. 2014). Interestingly, during the yeast-to-hyphae transition, there is not only an induction of genes required for the morphological transition *per se* (for example *UME6*, *EED1*,

and *HGC1*), but also there is an upregulation of hypha-specific virulence factors, including genes coding for adhesins (*HPW1, ALS3*), secreted aspartic proteases (*SAP4*, *SAP5*, *SAP6*), and the membrane-damaging toxin candidalysin (*ECE1*) (Mayer, Wilson et al. 2013, Wilson, Naglik et al. 2016).

The transition from the yeast to the hyphae form is promoted by environmental changes such as alkaline pH, the presence of serum, N-acetylglucosamine (Torosantucci, Angiolella et al. 1984), physiological temperature of the host (Odds 1988), both low O_2 and high CO_2 levels (Sudbery 2011, Lu, Su et al. 2013), and quorum sensing molecules (which can both promote and inhibit the hyphae formation) (Albuquerque and Casadevall 2012). In addition, *C. albicans* is able to change its morphology in response to starvation conditions: Iron deprivation leads to hyphae formation (Hameed, Prasad et al. 2008) and zinc deprivation to the generation of Goliath cells (Malavia, Lehtovirta-Morley et al. 2017).

Adhesion

C. albicans uses specific adhesin proteins to promote adherence to abiotic surfaces, to other microorganisms, or to host cells. These adhesion properties are required for pathogenesis (Verstrepen and Klis 2006, Garcia, Lee et al. 2011). The *C. albicans* Agglutinin-Like Sequence (ALS) gene family includes eight genes (*ALS1–7* and *ALS*9) (Hoyer, Green et al. 2008). Als3, the gene product of ALS family, and Hwp1, a hyphal associated cell surface protein, were found to be especially important for adhesion to host cells (Staab, Bradway et al. 1999, Phan, Myers et al. 2007, Wachtler, Wilson et al. 2011, Murciano, Moyes et al. 2012). Interestingly, adherence and metal homeostasis were described to be connected. For example, Als3 is not only an adhesin, but also a receptor, which is required for iron uptake, as it binds host ferritin, and this interaction is essential for iron acquisition from ferritin (Almeida, Brunke et al. 2008). Additionally, in response to long zinc deprivation, Goliath cells were found to have a hyper-adherent phenotype *in vitro* (Malavia, Lehtovirta-Morley et al. 2017), however, the reason for this increased adherence is not known so far.

Biofilm formation

The attachment of *C. albicans* cells to each other and to surfaces can result in the formation of biofilms. Biofilms are frequently formed on implanted medical devices and are highly resistant to antifungals and the host immune system, making biofilm-associated infections a severe clinical challenge (Nobile and Johnson 2015). *C. albicans* biofilms consist of yeast, pseudohyphal, and hyphal cells (Chandra, Kuhn et al. 2001). There are at least six TFs (Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1) that

are considered as the major regulators of biofilm development *in vitro* and *in vivo* (Nobile, Fox et al. 2012).

Trace metals have an influence on this virulence trait. Csr1 (an orthologue of Zap1 in *S. cerevisiae*), the main TF responsible for zinc homeostasis regulation, is a regulator of the biofilm maturation in *C. albicans*. The *csr1*Δ/Δ biofilms have an increased amount of yeast cells and accumulate significantly less farnesol (a quorum sensing molecule inhibiting hyphae formation) in comparison to wild type biofilms. The overexpression of *ZRT2* (coding for a membrane zinc importer the expression of which is activated by Csr1) in the *csr1*Δ/Δ strain restores both the yeast-hyphae ratio and the farnesol levels, indicating the importance of zinc assimilation for the biofilm maturation. In addition, it was suggested that the presence of zinc ions plays an important role in the cell to cell signaling events in biofilms (Ganguly, Bishop et al. 2011).

Hydrolases

C. albicans secretes proteases, phospholipases, and lipases. Members of the large secreted aspartic protease (Sap) family contribute to invasion and tissue damage (Felk, Kretschmar et al. 2002, Naglik, Challacombe et al. 2003, Dalle, Wachtler et al. 2010). Interestingly, Sap6 is able to degrade host tissues, leading to the release of host nutrients, including zinc ions, and thereby Sap6 facilitates zinc acquisition in *C. albicans* (Kumar, Breindel et al. 2017). There are large gene families encoding for secreted lipases (at least ten genes) and for phospholipases (four genes) in *C. albicans*. Secreted lipases, the expression of which is induced in several infection models, share 70% similarity in their amino acid sequence and seem to have a redundant function, as mutants lacking only single genes behaved similarly to the wild type strain both *in vitro* and *in vivo* (Hube, Stehr et al. 2000). Phospholipases hydrolyze ester linkages of glycophospholipids and promote tissue invasion; the presence of Plb1 and Plb5 was shown to be essential in a mouse systemic infection model (Leidich, Ibrahim et al. 1998, Theiss, Ishdorj et al. 2006).

pH-sensing and regulation

Host niches strongly differ in pH values ranging from extremely acidic in the stomach (pH < 2), acidic on the skin (pH 5.5), neutral in the blood (pH 7.4), and basic in some parts of the intestine (pH > 8.5) (Dubois 1932, Krahulec and Poncova-Kozouskova 1951, Arbenz 1952, Lang 1955). *C. albicans* is able to cope with a variety of pH levels during its commensal and pathogenic lifestyles. The changes in external pH levels cause the activation of the plasma membrane receptors Dfg16 and Rim21. This leads to the induction of a signaling cascade that governs the activation of

Rim101, a pH-responsive TF, *via* proteolytic cleavage (Davis 2009). In addition to the regulation of pH responsive genes, Rim101 is required for the expression of iron and zinc uptake genes under alkaline pH (Bensen, Martin et al. 2004), which can be explained by the fact that metals have a low solubility at alkaline pH.

C. albicans is not only able to survive under various pH levels, but can also actively modulate the extracellular pH. In the presence of glucose, acids are produced *via* glycolysis in *C. albicans*. However, under glucose limitation, *C. albicans* utilizes amino acids as a carbon source, which leads to the production of ammonia and thus to the alkalinization of the external environment. *C. albicans* mediated raise of the surrounding pH might trigger the yeast-to-hyphae transition, which is known to be crucial for its pathogenicity (Stewart, Hawser et al. 1989, Vylkova, Carman et al. 2011).

The presence of pH sensors, Rim101, and the alkalinization system are critical for *C. albicans* virulence (Davis, Edwards et al. 2000, Mitchell, Wu et al. 2007, Thewes, Kretschmar et al. 2007, Yuan, Mitchell et al. 2010).

Metabolic adaptation

C. albicans is able to rapidly change its metabolism depending on the niche it inhabits, due to its high metabolic plasticity. In blood, an environment with low levels of free metals (Martin, Savory et al. 1987), *C. albicans* activates its iron (Chen, Pande et al. 2011) and zinc uptake machinery (personal communications Philipp Kaemmer). Blood cells and macrophages efficiently recognize and phagocytose *C. albicans* cells. Inside the phagosomes *C. albicans* experiences various stressors, including nutrient deprivation, which *C. albicans* is able to counteract *via* reprograming its metabolism and using the glyoxylate cycle, gluconeogenesis, and/or β-oxidation of fatty acids (Lorenz and Fink 2001, Lorenz, Bender et al. 2004, Barelle, Priest et al. 2006, Piekarska, Mol et al. 2006, Ramirez and Lorenz 2007). *C. albicans* can escape phagocytes *via* hyphal formation and disseminate to various organs, where again different nutrient sources are available that *C. albicans* is able to successfully utilize. Thus, the metabolic flexibility gives *C. albicans* an opportunity to inhabit different host niches (Brock 2009, Fleck, Schobel et al. 2011).

Environmental stress response

Inside the host, *C. albicans* experiences heat, osmotic, oxidative, and nitrosative stresses. *C. albicans* senses the changing environment and further transmits signals *via* mitogen-activated protein (MAP) kinase pathways to specific TFs, which activate effector genes and generate a specific adaptive response. There are

three major MAP kinase signaling pathways that, depending on the stimuli, are activated *via* Mkc1-, Hog1-, or Cek1-MAP kinases.

C. albicans cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling pathway was shown to promote hyphal growth (Hogan and Sundstrom 2009) and recently, a link between the cAMP/PKA pathway and zinc in *C. albicans* was reported. Extracellular glucose or zinc can trigger cAMP spikes and intracellular zinc mobilization (Kjellerup, Winther et al. 2018). This "zinc flux" resembles events in mammalian cells, where zinc can serve as a second messenger (Yamasaki, Sakata-Sogawa et al. 2007), and the observations indicate that zinc might play a similar role in *C. albicans*. Thus, cAMP/PKA contributes to the detection and response to external zinc levels or carbon available sources (Kjellerup, Winther et al. 2018).

C. albicans possesses six heat shock proteins (HSPs) that are required to tolerate thermal stress and act as molecular chaperones in signaling pathways (Gong, Li et al. 2017). During host-induced elevated temperatures, low nutrient levels (including zinc limitation (manuscript in preparation Skrahina, Wilson et al.)), and accumulation of ROS, HSPs prevent the unfolded protein response (Richter, Haslbeck et al. 2010). The expression of HSP genes is mainly regulated by the TF Hsf1, which is essential for viability (Nicholls, MacCallum et al. 2011).

Following phagocytosis of fungal cells, the host accumulates ROS and Reactive Nitrogen Species (RNS) within the phagosome to inhibit fungal growth. To combat oxidative stress *C. albicans* relies on the catalase Cat1 and several SODs. *C. abicans* codes for six SODs in its genome, which all depend on metal cofactors: Cu/Zn-dependent Sod1 (cytosolic); Mn-dependent Sod2 (mitochondrial) and Sod3 (predicted to be cytosolic); and Cu-dependent Sod4, Sod5, and Sod6 (all extracellular). The flavorhemoglobin-related protein Yhb1 is critical for combating RNS in *C. albicans* (Hromatka, Noble et al. 2005). The ability of *C. albicans* to counteract both oxidative and nitrosative stresses is required for its pathogenicity (Hwang, Rhie et al. 1999, Hwang, Rhie et al. 2002, Hwang, Baek et al. 2003, Martchenko, Alarco et al. 2004, Frohner, Bourgeois et al. 2009).

Metal availability directly correlates with oxidative stress. Both iron overload and limitation leads to ROS accumulation. The absence of Cfl1, a ferric reductase, leads to an increased iron uptake *via* upregulation of other ferric reductase genes (*FRP1*, *CFL2,* and *FRE10)* and to the accumulation of intracellular iron in *C. albicans* cells. Consequently, a *cfl1*Δ/Δ mutant has elevated ROS levels (Xu, Qian et al. 2014). As iron plays a structural role in proteins, the deprivation of iron can lead to the unfolded protein response, which causes ROS accumulation (Malhotra and Kaufman 2007). The link between combating ROS under low iron levels can be seen in the case of Hap43, a

TF that regulates both iron homeostasis and oxidative stress resistance genes (Chakravarti, Camp et al. 2017). Zinc deprivation also leads to oxidative stress (Eide 2011) and *S. cerevisiae* cells were shown to have increased levels of ROS under zinc limitation conditions (Wu, Bird et al. 2007, Wu, Steffen et al. 2009). Additionally, we observed oxidative stress resistance response under zinc deficiency in *C. albicans* (manuscript in preparation Skrahina, Wilson et al.).

3.4. Metal acquisition

To counteract host-imposed iron and zinc deprivation, *C. albicans* relies on specific, often highly specialized, metal transporters and mobilization systems.

Reductive iron uptake

The *C. albicans* high-affinity reductive iron uptake system includes reductases, ferroxidases, and permeases.

The reductases contain FAD- and/or NAD-binding domains and are responsible for the reduction of the ferric iron to the ferrous iron state (De Luca and Wood 2000). *C. albicans* is predicted to possess 14 putative ferric reductases (Baek, Li et al. 2008, Almeida, Wilson et al. 2009). The expression of a range of reductases, including *CFL1*, *CFL2*, *CFL5*, *FRE9*, *FRP1*, and *FRP2*, has been shown to be activated by low iron levels (Bensen, Martin et al. 2004, Lan, Rodarte et al. 2004, Lee, Liu et al. 2005, Chen, Pande et al. 2011).

In the next step of iron uptake, ferroxidases re-oxidase the ferrous iron back to the ferric iron state to allow permeases to import this ferric iron into cells. *C. albicans* has four ferroxidases that require copper as a cofactor for their function (encoded by *FET3*, *FET31*, *FET33*, and *FET99*) and one copper-independent ferroxidase (encoded by *FET34*) (Almeida, Wilson et al. 2009). Following re-oxidation, iron is transported *via* plasma membrane permeases (Ftr1, Ftr2) into the cytoplasm or *via* vacuole membrane permeases (Fth1, Fth2) into the vacuole. Among the four permeases, the absence of the high affinity transporter Ftr1 leads to avirulence in a mouse model of systemic infection (Ramanan and Wang 2000). The ferroxidases and permeases act as complexes, and four ferroxidases (Fet3, Fet31, Fet34, and Fet99) can form complexes with both plasma and vacuole membrane permeases, while Fet33 only interacts with vacuolar permeases. The expression of *FTR1*, *FTH1*, *FET31*, *FET34,* and *FET99* is induced by low iron levels and the transcript levels of *FTR2* are regulated in an opposite manner. The expression of *FTH2*, *FET3*, and *FET33* does not depend on iron levels. The proper expression and function of permeases and ferroxidases is required to acquire iron, while preventing iron toxicity (Muzzey, Schwartz et al. 2013).

Siderophore-dependent iron uptake

While *C. albicans* itself does not produce siderophores, it is able to use xenosiderophores *via* a specific transporter, Sit1 (Heymann, Gerads et al. 2002, Haas 2003, Lan, Rodarte et al. 2004).

Host iron proteins as an iron source for *C. albicans*

C. albicans is able to utilize host proteins, including hemoglobin, hemin, ferritin, and transferrin, as an iron source.

The fungus binds hemoglobin in both yeast and hyphae forms (Moors, Stull et al. 1992, Santos, Buisson et al. 2003) *via* its hemoglobin receptors: Rbt5 and Pga7. From the surface, hemoglobin is transported into the cytoplasm and further delivered to the vacuole, where iron is extracted by the heme oxygenase Hmx1 (Pendrak, Chao et al. 2004). Similarly to hemoglobin, hemin utilization is Hmx1 dependent (Santos, Buisson et al. 2003). Ferritin utilization is only possible in the hyphal form and, as mentioned above, Als3 is required for binding of ferritin. It has been proposed that ferritin is denatured in the extracellular space and extracted iron is imported *via* the high affinity transporter Ftr1 (Almeida, Brunke et al. 2008). The utilization of iron from transferrin was found to depend on both Ftr1 and Fre10, a major cell-surface ferric reductase (Knight, Vilaire et al. 2005).

Iron mobilization

Because of high redox activity of iron, after the uptake, it is immediately distributed to its cellular targets and used in metabolic processes or stored for later use. Many iron-dependent processes, such as iron-sulfur protein (Fe-S) synthesis and the tricarboxylic acid (TCA) cycle with its iron-containing enzymes, take place in mitochondria, and indeed Mrs4, a mitochondrial iron importer, is essential for mitochondrial function (Xu, Cheng et al. 2012). Among many other functions, the vacuole is known for intracellular iron storage, detoxification, and mobilization. The presence of Ccc1, a vacuole iron importer, and Smf3, a vacuole iron exporter, was found to be essential for filamentation, adhesion, and virulence (Xu, Dong et al. 2014).

Iron homeostasis regulation

The transcriptional regulation of *C. albicans* iron homeostasis relies mainly on Sef1, Hap43, and Sfu1. During iron deficiency Sef1 and Hap43 activate iron uptake and repress iron consumption processes, and both factors have been found to be essential for virulence (Chen, Pande et al. 2011, Hsu, Yang et al. 2011, Singh, Prasad et al. 2011). In contrast, under elevated iron levels, Sfu1 represses genes responsible

for iron uptake. Accordingly, Sfu1 was shown to be required for commensalism in the gut environment, which is considered to have comparatively high iron levels. Sef1, Hap43, and Sfu1 regulate each other's expression in a feed-forward loop system (Chen, Pande et al. 2011).

Homologs of Hap43 that perform iron homeostasis regulation exist in pathogenic fungi, including *Aspergillus nidulans*, *A. fumigatus*, and *Fusarium oxysporum.* Moreover, the constituent domains of Hap43 are generally conserved, as is the role of Hap43 in regulating iron homeostasis under starvation among these fungi. However, in *A. nidulans*, *A. fumigatus*, and *F. oxysporum* Hap43 mediates the response to iron overload (Gsaller, Hortschansky et al. 2014), which we found not to be the case in *C. albicans* (Skrahina, Brock et al. 2017).

Zinc uptake machinery

In order to obtain zinc from the extracellular environment, *C. albicans* relies on the zinc scavenger (named "zincophore") and receptor system, as well as the plasma membrane transporter Zrt2. The "zincophore" system consists of the plasma membrane zinc importer Zrt1 and the secreted protein Pra1, which binds zinc with high affinity. Zinc-loaded Pra1 then interacts with Zrt1 to deliver the metal into the cytoplasm. The "zincophore" machinery is upregulated under alkaline pH and zinc limitation, and its presence is required for host cell damage in the tissue culture infection model (Citiulo, Jacobsen et al. 2012). So far the proven "zincophore" system was found only in *C. albicans*, however *A. fumigatus* encodes orthologues of *ZRT1* and *PRA1* and *B. dermatitidis* - orthologue of *PRA1*, in both species these genes are highly expressed during the systematic infection model in mice (Amich, Vicentefranqueira et al. 2010, Citiulo, Jacobsen et al. 2012, Munoz, Gauthier et al. 2015). The presence of Zrt2 is essential for zinc assimilation from the extracellular milieu at acidic pH and low zinc levels. Mice infected with *zrt2*Δ/Δ have a reduced fungal burden in comparison to control animals infected with the wild type strain (Crawford, Lehtovirta-Morley et al. 2018).

Zinc mobilization

Similar to iron, *C. albicans* has mechanisms to counteract elevated zinc levels, mainly by storing and detoxifying zinc in zincosomes, vesicular zinc storage compartments, and vacuoles. Zrc1 is a zincosome zinc importer, which is essential for liver colonization during infection, indicating a high level of zinc in this organ (Crawford, Lehtovirta-Morley et al. 2018). It was shown that upon extracellular zinc deprivation, *C. albicans* induces the expression of *ZRT3*, an orthologue of *S. cerevisiae* vacuolar

zinc exporter (manuscript in preparation Skrahina, Wilson et al.), which indicates that the pathogen mobilizes its zinc storage from the vacuole during times of zinc starvation.

Zinc homeostasis regulation

In contrast to the complex regulation of iron homeostasis, the regulation of genes essential for zinc sequestration, storage, and detoxification seems to be mainly controlled by a single TF, Csr1 (Nobile, Nett et al. 2009). In addition, Csr1 regulates genes essential to cope with zinc deprivation-associated stresses, like *SOD3,* the product of which contributes to oxidative stress resistance (Nobile, Nett et al. 2009). Recently, Sut1 was shown to contribute to the positive regulation of zinc homeostasis genes *in vivo* (Xu, Solis et al. 2015). The absence of Csr1 and Sut1 leads to reduced virulence, which in the case of *sut1*Δ/Δ can be reverted by overexpression of *ZRT2* (Xu, Solis et al. 2015). The pH-dependent regulation of the zincophore system is mediated by Rim101 (Bensen, Martin et al. 2004, Xu, Solis et al. 2015). Additionally, we identified Ssn6 as a novel player in zinc homeostasis regulation (manuscript in preparation Skrahina, Wilson et al.). In *C. albicans*, Ssn6 is known to be a regulator of hyphal growth and white-opaque switching (Hwang, Oh et al. 2003, Garcia-Sanchez, Mavor et al. 2005). We found Ssn6 to be required for the activation of zinc uptake and mobilization processes under zinc deprivation (manuscript in preparation Skrahina, Wilson et al.).

3.5. Aims of the study

Despite therapeutic advances, *C. albicans* remains a common cause of severe fungal infections. The current antifungal therapy against invasive systemic candidiasis has important drawbacks, including toxicity to the host and the development of fungal resistance. Vaccines are sometimes considered as a novel approach in preventing *Candida* infections and improving the treatment outcome. However, so far, no vaccines exist against *C. albicans* (Wang, Sui et al. 2015), and the inherent problem of developing a vaccine against a commensal microorganism should be considered. To circumvent some of these problems, it has been suggested to target hyphae-associated proteins, which are thought to be present mainly during the pathogenic phase (Jacobsen, Wilson et al. 2012). Given the importance of metal acquisition in pathogenesis, the *C. albicans* metal uptake machinery is a potential drug and/or vaccine target. Metal transporters are generally located on the cell surface and are therefore accessible. Additionally the “zincophore” is a secreted zinc chelator, which is unique to certain fungal species. Ferritin-binding Als3, which is only expressed in the hyphal stage, has been suggested as a vaccine target, although at that time, its role in iron uptake was unknown (Spellberg, Ibrahim et al. 2006, Spellberg, Ibrahim et al. 2008). Therefore, the *C. albicans* metal utilization system might hold further candidates for novel therapeutic approaches.

Although our understanding of *C. albicans* metal acquisition systems and their contributions to virulence has recently been expanded (Gerwien, Skrahina et al. 2018), many aspects remain unclear. Therefore the focus of this thesis was to analyze *C. albicans* iron and zinc homeostasis in response to metal fluctuations in detail using comprehensive transcriptome and mutant screening approaches.

While the strategies of *C. albicans* to cope with iron deficiency are comparatively well described, the response of *C. albicans* to iron overload has not been fully investigated. Therefore, the aim of this work was to analyze the role of *C. albicans* TFs in mediating the fungal response to iron overload when compared to other fungal species (Skrahina, Brock et al. 2017)

In contrast to iron, *C. albicans* zinc homeostasis is poorly investigated. In the present thesis, the aim was to characterize the transcriptional response to zinc limitation in both *C. albicans* wild type and *zrt2*Δ/Δ strains. Additionally, we planned to detect novel contributors to zinc homeostasis regulation and thus designed a transcription factor deletion library screen under low zinc levels (manuscript in preparation Skrahina, Wilson et al.). Adding to the investigation of the regulatory side, we aimed to analyze *in vitro* and *in vivo* the effectors, like the membrane zinc uptake

machinery and the intracellular zinc distribution systems (Crawford, Lehtovirta-Morley et al. 2018).

4. Manuscripts

4.1. Manuscript I: Skrahina *et al*., *Frontiers in Microbiology*, 2017

Candida albicans Hap43 domains are required under iron starvation but not excess

Volha Skrahina, Matthias Brock, Bernhard Hube, Sascha Brunke

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Summary:

As a successful opportunistic and commensal fungus, *Canida albicans* inhabits host niches with various iron concentrations. In order to do so, *C. albicans* relies on a transcriptional network that regulates iron assimilation and utilization processes. Hap43 is a transcriptional factor that, among other functions, is essential for both activating iron uptake machinery and repressing iron utilization pathways under iron deficiency. Recently, the ortholog of Hap43 in *Aspergillus fumigatus,* HapX, was shown to be required for iron homeostasis regulation under iron excess conditions, in addition to its previously described functions under iron limitation. Hap43 and HapX have similarities in their amino acid sequence, domains organization, and the function under low iron levels. However, in contrast to HapX, Hap43 is not essential for growth under sufficient iron levels in *C. albicans*. This result demonstrates the distinct strategies that fungi have evolved in order to cope with the same stressor.

Own Contribution:

Volha Skrahina designed the study, performed all of the experiments, evaluated and interpreted results, generated figures, and wrote the manuscript. The following experiments were carried out in the laboratory: construction of the plasmids and strains, phenotypic profiling, and qRT-PCRs. The other authors suggested experiments, interpreted results, and revised the manuscript.

Estimated authors' contributions:

Volha Skrahina	**60%**
Matthias Brock	10%
Bernhard Hube	5%
Sascha Brunke	25%

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Candida albicans Hap43 Domains Are Required under Iron Starvation but Not Excess

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Iron availability is a central factor in infections, since iron is a critical micronutrient for all living organisms. The host employs both iron limitation and toxicity strategies to control microbial growth, and successful pathogens are able to tightly coordinate iron homeostasis in response to changing iron levels. As a commensal and opportunistic pathogen, *Candida albicans* copes with both iron deficiency and excess *via* the precise regulation of iron acquisition, consumption and storage. The *C. albicans* transcription factor Hap43 is known to be required for the iron starvation response, while specific domains of its ortholog, HapX, in *Aspergillus fumigatus*, were recently shown to regulate iron uptake and consumptions genes under both low and high iron levels. Therefore, we investigated the contribution of *C. albicans* Hap43 domains in response to changing iron levels. We found the C-terminus of Hap43 to be essential for the activation of iron uptake genes during iron starvation, whereas, in contrast to *A. fumigatus*, Hap43 was not required in mediating adaptation to iron resistance. These data indicate that the generally conserved metal acquisition systems in fungal pathogens can show individual adaptations to the host environment.

Keywords: iron homeostasis, iron deficiency and toxicity, transcriptional regulation, hemoglobin, fungal pathogenicity

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INTRODUCTION

Iron is a trace metal with crucial roles in a multitude of biological processes such as oxidative phosphorylation, oxygen transport, and oxidative stress detoxification. Thus, iron availability is recognized as a central factor in infections: the host restricts access to iron in order to prevent microbial growth in a process known as "nutritional immunity" (Weinberg, 1975), while pathogens employ various strategies to obtain the metal from the host (Hood and Skaar, 2012). However, an excess of iron can become toxic due to the production of hydroxyl radicals in the Fenton reaction (Halliwell and Gutteridge, 1984). In addition to iron limitation, the host employs the toxic properties of iron to control infections. Therefore, pathogens have evolved to cope not only with iron limitation, but also with high iron levels (Chen et al., 2011; Gsaller et al., 2014; Xu et al., 2014; VanderWal et al., 2017). In conclusion, a successful pathogen requires mechanisms that tightly regulate iron homeostasis.

As a commensal and opportunistic pathogen, *Candida albicans* is able to cope effectively with both iron limitation and excess. In order to survive within the host during severe iron limitation,

C. albicans activates its iron acquisition machinery and represses iron consumption pathways (Lan et al., 2004; Chen et al., 2011). Iron acquisition in *C. albicans* encompasses the use of the host's iron transport and storage proteins; acquisition of (xeno-)siderophores (produced by other microorganisms, as *C. albicans* does not synthesize its own); and direct import *via* its high-affinity reductive iron uptake system. In the human body 70% of iron is contained in heme, which is mainly found in hemoglobin (Doherty, 2007), and which *C. albicans* can use as a source of iron (Moors et al., 1992; Santos et al., 2003). *C. albicans* hemoglobin utilization relies on the extracellular heme receptors, Rbt5 and Pga7, as well as the heme oxygenase Hmx1 (Kulkarni et al., 2003; Pendrak et al., 2004; Kuznets et al., 2014). Iron bound to xeno-siderophores is sequestered *via* Sit1, a siderophore-specific membrane importer (Heymann et al., 2002; Hu et al., 2002). The high-affinity reductive uptake system finally consists of reductases, which reduce Fe^{3+} to Fe^{2+}; ferroxidases, responsible for re-oxidation to Fe^{3+}, and permeases, which import Fe^{3+} into the cell (Ramanan and Wang, 2000; Chen et al., 2011). Under iron excess, *C. albicans* down-regulates iron acquisition systems and up-regulates iron-requiring processes such as biosynthesis of Fe-S clusters, heme, and heme-containing enzymes, respiration, and the tricarboxylic acid (TCA) cycle (Lan et al., 2004). Additionally, under iron excess, Ccc1, a vacuole iron importer, is induced and iron is transported into the vacuole and thereby rendered harmless to the cell (Xu et al., 2014).

C. albicans iron homeostasis was shown to be tightly regulated depending on the environment's iron availability. Under high iron levels, Sfu1, a GATA family transcription factor (TF), represses iron acquisition genes and Sef1, a Zn(2)Cys(6) transcription factor. Under low iron levels, Sef1 in turn activates iron uptake genes and Hap43, a part of the CCAAT-binding complex (CBC), which then represses Sfu1 and genes involved in iron-dependent processes (Chen et al., 2011). Hap43 carries out its function in the regulation of gene expression *via* its interaction with the CBC (Baek et al., 2008). The CBC itself is a conserved heterotrimeric DNA-binding complex present in fungi, plants, and mammals (Mantovani, 1999). Hap43 homologs, which mediate the gene regulation after CBC binding to DNA, can however only be found in fungi. In *Aspergillus nidulans* (HapX), *A. fumigatus* (HapX), *Cryptococcus neoformans* (HapX), and *C. albicans* (Hap43), the Hap/CBC complex mediates both positive and negative gene regulation in response to changing iron levels. Consequently, Hap43 is required for virulence in *A. fumigatus*, *C. neoformans*, and *C. albicans* (Schrettl et al., 2008, 2010; Jung et al., 2010; Hsu et al., 2011; Singh et al., 2011).

The Hap43 domains found in *C. albicans* are conserved among pathogenic fungi (Gsaller et al., 2014). The N-terminus includes the CCAAT-binding, b(ZIP), and coiled-coil domains, while the C-terminus consists of three cysteine-rich regions (CRR, each with four cysteine (Cys) residues), designated A, B, and C, and a single cysteine (**Figure 1**). Interestingly, *A. fumigatus* HapX was recently found to be essential under both iron limitation and excess (Gsaller et al., 2014). The HapX C-terminus is essential for growth during iron starvation, *via* the activation of the iron uptake gene (*mirB*) and the repression of iron consuming processes. In contrast, the highly conserved CRR-A, CRR-B, and—to a lesser degree—CRR-C domains of HapX allow growth under iron excess *via* the activation of genes required for vacuolar iron sequestration (*cccA*) and iron consumption by iron-sulfur cluster containing enzymes (*leuA*) and heme biosynthesis (*hemA*) (Gsaller et al., 2014).

C. albicans Hap43 is known to be essential during iron starvation (Baek et al., 2008; Chen et al., 2011; Hsu et al., 2011), but in contrast to *A. fumigatus*, the contributions of the individual Hap43 domains have not been investigated. Here, we studied the role of each individual Hap43 domain and found that, similar to *A. fumigatus*, the C-terminus of Hap43 is essential for the activation of iron uptake genes. Since *A. fumigatus* HapX mediates resistance against high iron levels, we investigated the function of *C. albicans* Hap43 under similar conditions. Surprisingly, we found that Hap43 appears to play only a minor role under iron excess in *C. albicans*, in stark contrast to *A. fumigatus*.

MATERIALS AND METHODS

Media and Growth Conditions

C. albicans strains were routinely grown in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose). Transformants were selected on SD medium (0.67% yeast nitrogen base [Difco], 2% glucose, 2% Oxoid agar) which was supplemented with the appropriate amino acids and/or uridine. For phenotypic profiling under iron starvation, cells were grown in SD (0.69% yeast nitrogen base without iron [Formedium], 2% glucose). For transcriptional profiling under iron starvation, cells were grown in SD (0.69% yeast nitrogen base without iron [Formedium], 2% glucose, Bathophenanthrolinedisulfonic acid disodium salt (BPS) 0.5 mM [Alfa Aesar]). For phenotypic profiling under high iron levels in liquid media, cells were grown at 30° in SD buffered to pH 4.0 (0.69% yeast nitrogen base without iron [Formedium], 2% glucose, citric acid 59 mM, sodium citrate 41 mM, $FeCl_3$ 30 or 2 mM). For phenotypic profiling under high iron levels on solid media, cells were grown at 30° on SD buffered to pH 4.0 (0.69% yeast nitrogen base without iron [Formedium], 2% glucose, 2% Oxoid agar, citric acid 59 mM, sodium citrate 41 mM, $FeCl_3$ 5 mM (the highest iron concentration which allowed the medium to solidify). For hemoglobin drop tests, cells were grown at 37° on SD buffered to pH 7.6 (0.69% yeast nitrogen base without iron [Formedium], 2% glucose, 2% Oxoid agar, citric acid 6.35 mM, Na_2HPO_4 187.3 mM, 0.07 mg/ml hemoglobin from bovine blood [Fluka], BPS 0.5 mM [Alfa Aesar]).

Plasmid and Strain Construction

The *C. albicans* strains used in this study are listed in Table S2. Plasmids used in this study were constructed using the In-Fusion HD cloning kit [Takara Bio USA] and checked *via* restriction enzyme digestion, PCR, and Sanger sequencing using the primers listed in Table S3. Deletion strains produced in this study were generated in the BWP17 background (Wilson et al., 1999) as previously described (Gola et al., 2003; Walther and Wendland, 2003). All strains were verified by colony PCR. Primers used for mutant production and verification are listed in Table S3. Complementation plasmids were generated by

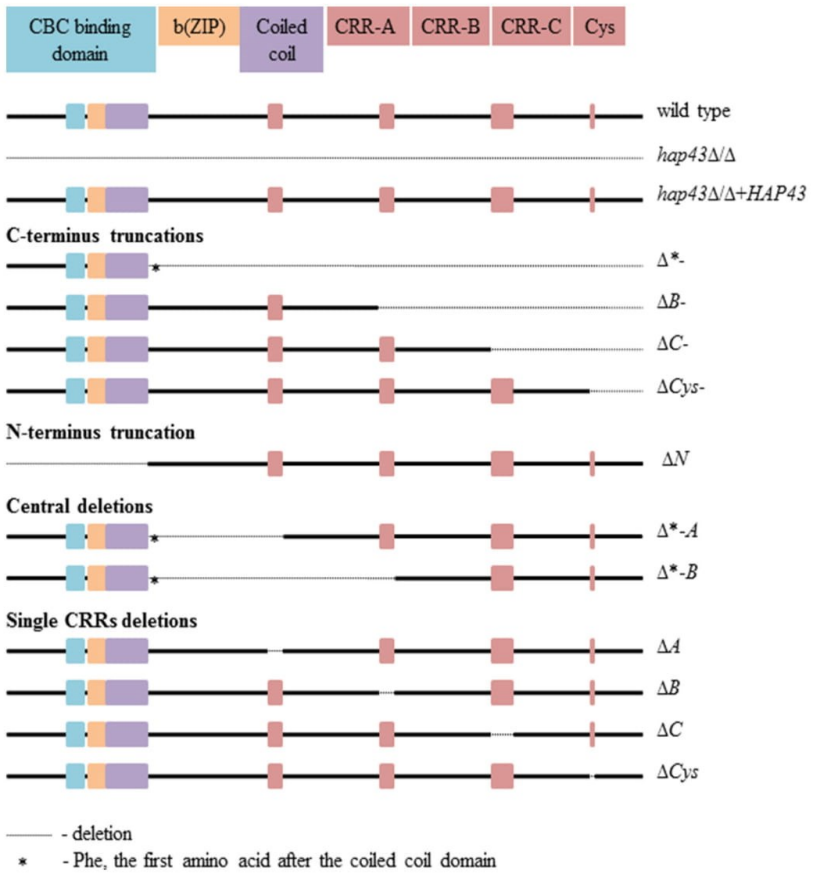

FIGURE 1 | The Hap43 domains organization in the wild type and 13 mutants.

amplifying the gene of interest, including the upstream and downstream intergenic regions followed by cloning into CIp10 (Murad et al., 2000). Resultant complementation constructs were linearized with *Stu*I and used to transform strains as stated above.

Growth Curve Analyses

For iron starvation phenotypic profiling, strains were grown in YPD overnight, washed four times in nanopure water and inoculated to OD 0.005 in SD without iron for a first round of starvation to largely deplete internal iron storage. Afterwards, the cells were washed again in nanopure water and inoculated to OD 0.005 in SD without iron for the second starvation phase. During this time, they were placed in the reader [TECAN infinite M200Pro], grown at 30°C, and OD 600 was determined after 30 s of orbital shaking every 30 min. For phenotypic profiling under high levels of iron, the lag phase yeasts were washed only once.

PCR and Quantitative Real-Time Reverse Transcription-PCR (qRT-PCR)

To determine gene expression levels, cells were grown in YPD overnight (30°C, 180 rpm) and then washed four times with nanopure water. All tested strains were inoculated to OD 0.2 either in 10 ml iron-free SD with additional BPS 0.5 mM, or in iron-free SD supplemented with $FeCl_3$ 20 mM for 4 h. For the shift assay, strains were grown in YPD overnight, washed four times in nanopure water, inoculated to OD 0.2 in iron-free SD with additional 0.5 mM BPS and incubated for 4 h. For the shift, these strains were washed once in nanopure water and inoculated to OD 0.2 in iron-free SD supplemented with $FeCl_3$ 50 μM and incubated for 30 min. Then, the yeasts were frozen in liquid nitrogen, followed by a total RNA extraction [Qiagen RNeasy]. The total RNA was treated with DNase [Epicenter Baseline-ZERO] and purified using the kit [Qiagen RNeasy]. RNA quality was verified *via* the bioanalyzer instrument [Agilent]. The RNA concentration was determined using a NanoQuant plate in

the reader [TECAN infinite M200Pro]. For each sample, 500 ng RNA was transcribed into cDNA, which was checked *via* PCR for genomic DNA (gDNA) contamination using intron-spanning amplicons (Table S3). In addition, standard PCRs with cDNAs from wild type and mutant strains as template for *HAP43* amplification were performed (**Figure S1**). Finally, a total amount of 1.85 ng cDNA was used for each qRT-PCR using the fluorescent dye EvaGreen [Bio&Sell] in the thermal cycler [CFX96^{TM} Real-Time System Bio-Rad] in biological and technical triplicates. Expression rates were determined relative to the housekeeping gene *ACT1* and analyzed using the Software [Bio-Rad CFX Manager]. For the shift assay the qRT-PCR was performed in five biological replicates, each with three technical replicates, and normalized to the transcript levels of *ACT1* in each strain and to the wild-type transcript levels. All primers are listed in Table S3. Statistical analyses were performed by one way ANOVA followed by Dunnet tests, where the mean of each mutant strain was compared with the mean of the wild type strain.

RESULTS

Mutated *Hap43* Variants Are Transcribed under Iron Depletion

In order to dissect the individual functions of the different Hap43 domains, we created 13 mutant strains: a full-length *hap43*Δ/Δ deletion and a *hap43*Δ/Δ+*HAP43* complemented strain; C-terminal truncations covering the CRRs (Δ*-, Δ*B*-, Δ*C*-, and Δ*Cys*-); an N-terminally truncated strain (Δ*N*); deletions of central parts of Hap43 (Δ*-*A* and Δ*-*B*); and finally targeted deletions of the CRRs only (Δ*A*, Δ*B*, Δ*C*, and Δ*Cys*). The locations of all deletions are shown in **Figure 1**, and the genetic identity of all strains was checked by Sanger sequencing of the *HAP43* locus. The *HAP43* variants were actively transcribed in all strains, except, of course, for *hap43*Δ/Δ, as evidenced by specific PCR amplicons obtained from cDNAs of all strains grown under low iron levels (**Figure S1**).

Both C- and N-Terminus, but Not the Cysteine Residues, Are Essential for Hap43 Function under Low Iron

Having established the expression of all *HAP43* variants, we went on to investigate the growth of wild type and mutant strains in an iron-free medium (SD w/o Fe). A 24 h pre-starvation in the absence of iron was performed in order to deplete the internal iron storage. As expected (Baek et al., 2008), *hap43*Δ/Δ exhibited a growth defect under this iron-depleted condition in comparison to both wild type and *hap43*Δ/Δ+*HAP43* complemented strains (**Figure 2A**). Mutants truncated at the C-terminus, N-terminus, and deletions of the central regions (to a lesser degree than C- and N-truncations) showed growth defects in SD w/o Fe (**Figures 2B–D**). In contrast, the mutants lacking the A, B, or C CRRs or the individual Cys retained wild type-like growth during iron depletion (**Figure 2E**), indicating the dispensability of these regions for regulation of iron uptake.

Hemoglobin Utilization Requires Both the C- and N-Terminus of Hap43

We performed a transcriptional screening of iron-related genes after 8 h iron limitation in both wild type and *hap43*Δ/Δ *via* qRT-PCR. All expression levels were normalized to gene expression levels of the same gene in wild type cells grown in YPD (a medium with sufficient iron). These analyses revealed *RBT5* as one of the most highly up-regulated genes in the wild type upon iron starvation, with a more than 30-fold increase in mRNA abundance. This induction was severely reduced, albeit still present at low levels in the *hap43*Δ/Δ strain (Table S1). Hap43 has previously been shown to positively regulate the expression of *RBT5*, *PGA7*, and *HMX1*, the key players of the hemoglobin utilization machinery, during iron deprivation (Chen et al., 2011; Singh et al., 2011; Kuznets et al., 2014). In agreement, the expression of *PGA7* and *HMX1* was down-regulated in the *hap43*Δ/Δ strain in comparison to the wild type in our screening. Therefore, we focused in more detail on the regulation of the hemoglobin utilization pathway genes in the wild type and all mutant strains.

The expression levels of *RBT5*, *PGA7*, and *HMX1* were measured after 4 h of growth under iron depletion and normalized to the transcript levels of *ACT1* in each strain (**Figure 3**). Truncation of both the C- or the N-terminus of Hap43 led to a reduced induction of *RBT5*, *PGA7*, and *HMX1* transcript levels compared to the wild type (**Figures 3A–C**), in agreement with the growth defect seen under iron depletion for these strains (**Figures 2B,C**). Surprisingly, deletions of the central regions of Hap43 did not abolish the induction of hemoglobin up-take genes (**Figures 3A–C**), and *HMX1* transcript levels were even higher in the Δ*-*A* strain, than in the wild type (**Figure 3C**), although these strains (Δ*-*A* and Δ*-*B*) exhibited similar growth defects under iron-deficient conditions (**Figure 2D**). Finally, all single CRR deletions did not change the regulation of the hemoglobin up-take machinery genes, with the exception of the Δ*B* strain, where *RBT5* transcription was slightly reduced compared to the wild type (**Figure 3A**)—mirroring the dispensability of the CRRs in our iron-depleted medium (**Figure 2E**).

To investigate whether the mutants are still able to use hemoglobin as an iron source, all strains were pre-starved for 24 h in the absence of iron and shifted to a medium with hemoglobin as the sole source of iron. C- and N-terminal truncations led to an inability to grow with hemoglobin alone, whereas single CRRs deletions had no effect on growth (**Figure 4**), in agreement with our transcriptional data. Strains with deletions of central domains of Hap43 showed different phenotypes: the Δ*-*A* strain remained able to use hemoglobin as an iron source, whereas the Δ*-*B* strain displayed a severe growth defect.

Hap43 Is Not Required for Growth or Gene Regulation under Iron Excess

In *A. fumigatus*, HapX is essential in both low and excessively high iron environments (Gsaller et al., 2014). However, the functional role of *C. albicans* Hap43 in adaptation to high iron has not yet been investigated in detail. We therefore tested the

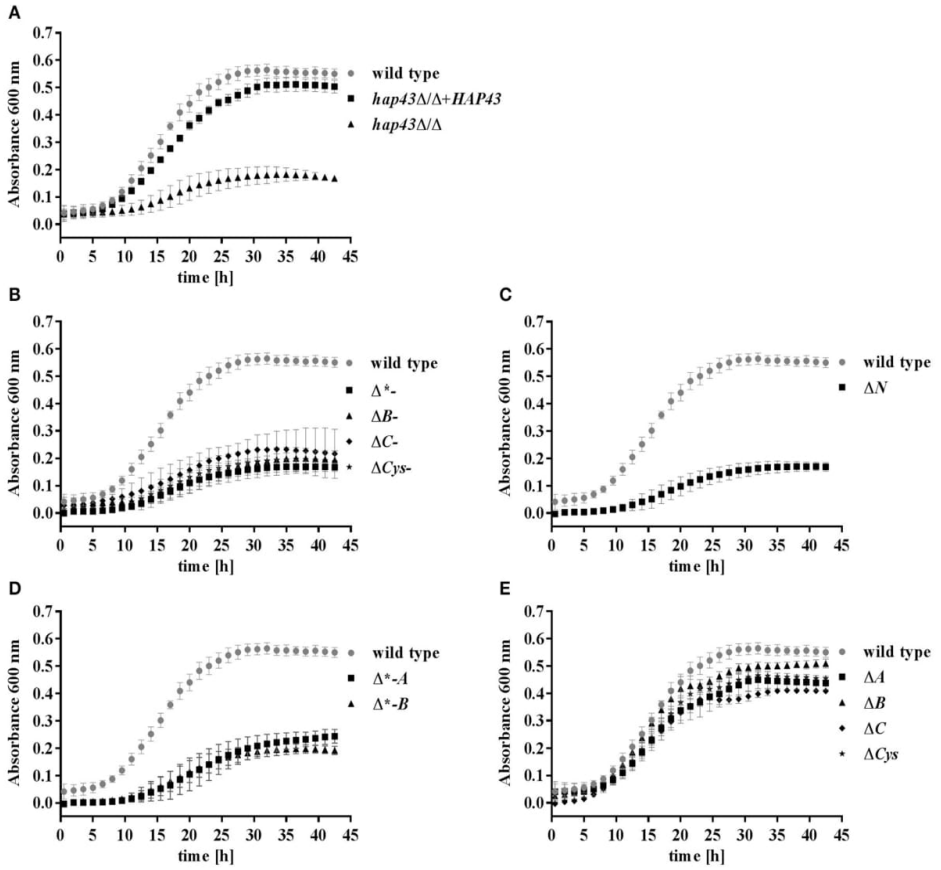

FIGURE 2 | Phenotypic profiling of the wild type and mutant strains under iron limitation. Strains were pre-starved 24 h for iron, and then grown in SD w/o iron with a starting OD of 0.005. **(A)** Wild type, *hap43*Δ/Δ, *hap43*Δ/Δ+*HAP43*; **(B)** C-terminus truncations: Δ*-, Δ*B*-, Δ*C*-, and Δ*Cys*-; **(C)** the N-terminus truncation: Δ*N*; **(D)** central deletions: Δ*-*A* and Δ*-*B*; **(E)**; and single CRR deletions: Δ*A*, Δ*B*, Δ*C*, Δ*Cys*.

ability of *hap43*Δ/Δ to grow under iron excess on solid media. The *sfu1*Δ/Δ strain was included as a control, as it was previously shown that Sfu1 represses iron up-take genes in the presence of high levels of iron (Lan et al., 2004; Chen et al., 2011). As expected, *sfu1*Δ/Δ was sensitive to elevated environmental iron. However, *hap43*Δ/Δ grew nearly identically to the wild type on this medium (**Figure 5A**). We therefore tested growth in liquid medium containing 2 and 30 mM ferric iron (**Figure 5B**). As expected, the growth of all strains was reduced under iron excess, but we found no difference in the growth of wild type, *hap43*Δ/Δ, CRRs deletion (Δ*A*, Δ*B*, Δ*C*, Δ*Cys*) and the C-terminal truncation (ΔC-) strains. A deletion of *SEF1*, which is known to control *HAP43* expression under iron limitation (Chen et al., 2011) was similarly without visible effect. Only *sfu1*Δ/Δ, which was included as a control for iron excess stress, was defective in growth (**Figure 5B**). These data suggest that, unlike *A. fumigatus* HapX, *C. albicans* Hap43 or its individual domains are not involved in the response to elevated environmental iron.

To further investigate this hypothesis, the mRNA levels of several genes involved in iron homeostasis were measured in wild type, *hap43*Δ/Δ, and *sfu1*Δ/Δ cells under conditions of iron excess. Expression analyses included the determination of transcription factor genes (*HAP43*, *SEF1*, and *SFU1*), genes essential for the reductive iron uptake (*FRE9*, *FRP1*, *FTH1*, *FTR1*, and *FTR2*), hemoglobin utilization (*RBT5*, *PGA7*, and *HMX1*), siderophore transport (*SIT1*), and vacuolar iron import (*CCC1*) (Xu et al., 2014). Deletion of the *SFU1* repressor gene resulted in an inappropriate overexpression of all iron uptake genes even under iron excess (**Figure 6A**). These data are in agreement with previous findings (Lan et al., 2004) and may explain the severe growth defect of *sfu1*Δ/Δ under excessive iron (**Figure 5**). In stark contrast, *hap43*Δ/Δ exhibited an expression profile of iron

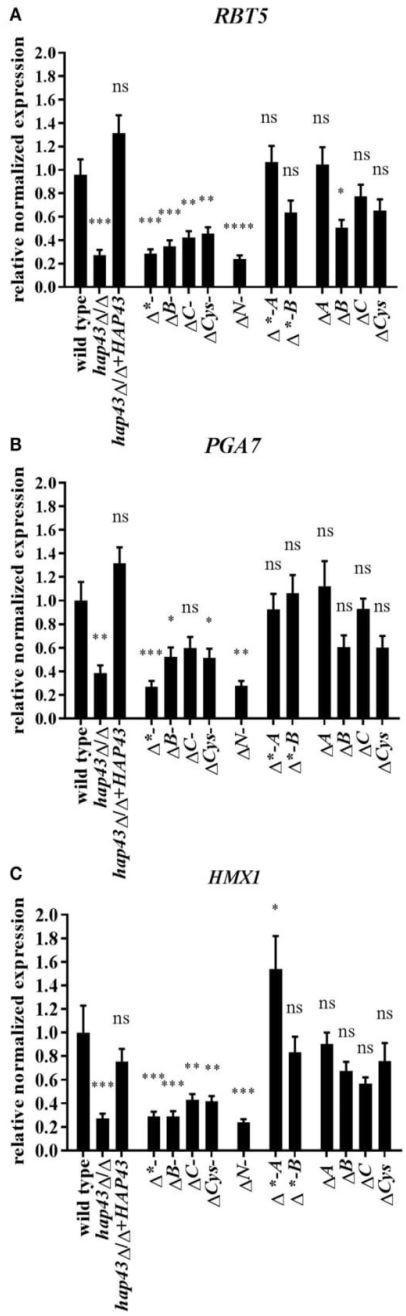

FIGURE 3 | Transcriptional profiling of hemoglobin uptake machinery genes under iron starvation. *RBT5* **(A)**, *PGA7* **(B)**, and *HMX1* **(C)** transcript levels were measured by qRT-PCR in wild type and all mutant strains. The expression was normalized to the transcript levels of *ACT1* in each strain. Asterisks indicate statistical significance compared to the wild type strain (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ns, $p > 0.05$).

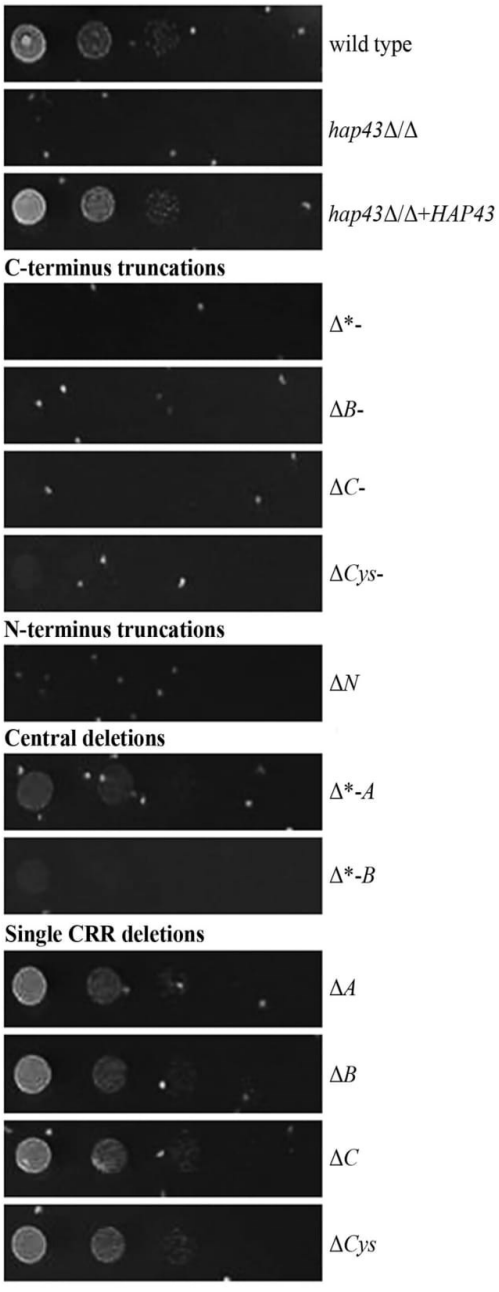

FIGURE 4 | Phenotypic profiling of the wild type and mutant strains in the presence of hemoglobin as the sole iron source. Strains were pre-starved 24 h for iron, and then the drop test was performed at 37° on SD (buffered to pH 7.6 and hemoglobin as the sole source of iron) and on YPD, where all strains retained wild type-like growth (not shown).

uptake genes very similar to the wild type, except for *CCC1,* which was significantly less transcribed in *hap43*Δ/Δ in comparison to the wild type. This supports our notion that Hap43 does not play a major role in *C. albicans* transcriptional regulation under conditions of high iron.

Genes with roles in iron utilization are generally transcriptionally induced by *C. albicans* in high-iron environments (Lan et al., 2004). We therefore analyzed transcript levels of iron consuming genes in wild type, *hap43*Δ/Δ, and *sfu1*Δ/Δ under steady-state iron excess. Genes coding for aconitase (*ACO1*), heme biosynthesis (*HEM3* and *HEM4*) and for heme-containing proteins (*CCP1*, *CYC1*, and *CYT1*) were all significantly up-regulated in *sfu1*Δ/Δ, whilst their expression levels in *hap43*Δ/Δ did not differ from the wild type (**Figure 6B**). Therefore, in the absence of Sfu1, both iron uptake and iron consumption genes are misregulated, which is in accordance with the severe growth defect of *sfu1*Δ/Δ under high iron growth conditions. However, Hap43 is dispensable for gene regulation under conditions of toxic iron levels (**Figure 6**), which contrasts the bifunctional role of *A. fumigatus* HapX that is important under conditions of iron starvation and iron saturation.

In *A. fumigatus* the lack of HapX abolishes the transcription of genes coding for iron-requiring processes (*acoA*, *cycA*, *leuA*, *hemA*) especially upon a shift from iron-limited to iron-replete conditions (Gsaller et al., 2014). We therefore investigated the transcript levels of *C. albicans* iron consumption genes (*ACO1*, *CCC1*, *CCP1*, *CYC1*, *CYT2*, *HEM3*, *HEM4*, and *ISA1*) after a shift from a 4 h starvation pre-culture to iron-replete conditions. In contrast to *A. fumigatus*, only two of all genes tested (*ACO1* and *CYC1*) were about 2-fold less transcribed in the *hap43*Δ/Δ mutant (Gsaller et al., 2014) (**Figure S2**). Expression levels of all other decreased <2-fold or were identical to wildtype transcript levels (**Figure S2**).

DISCUSSION

Since both iron starvation and excess are harmful to microbial life, all pathogenic organisms must have developed sophisticated mechanisms to cope with iron fluctuations within the host. Under iron deficiency *C. albicans* relies on the transcription factors (TFs) Sef1 and Hap43 to control the expression of iron uptake and consumption genes. Consequently, these are required for full virulence (Chen et al., 2011; Hsu et al., 2011; Singh et al., 2011). In environments of adequate or elevated iron, another factor, Sfu1, governs the repression of iron uptake genes and thus, is essential for commensal growth in the murine gut, a niche thought to be predominantly iron-replete. On the other hand, the gut niche can rapidly change to become an iron limiting environment through microbial competition and by food intake, and consequently Sef1 was also shown to be important for *C. albicans* commensal growth, although to a lesser degree than Sfu1 (Chen et al., 2011). In contrast to *C. albicans*, the filamentous fungi *A. fumigatus*, *A. nidulans*, and *F. oxysporum*, which do not preferentially colonize human mucosal surfaces, lack Sef1, and iron homeostasis is thought to be maintained by two other TFs. One of these TF is HapX, the Hap43 homolog, which controls gene expression under both low and high iron levels and therefore is essential for virulence (Schrettl et al., 2008, 2010; López-Berges et al., 2012; Gsaller et al., 2014). The second factor is SreA, a Sfu1 homolog, which additionally represses iron uptake genes under high iron levels (Haas et al., 1999; Schrettl et al., 2008). In-depth analysis of *A. fumigatus* HapX domains, which are conserved among the fungi, have shed light on how a single TF is able to both repress and activate genes depending on the iron content of the surrounding environment (Gsaller et al., 2014). Here, we dissected the role of *C. albicans* Hap43 domains in gene regulation in response to different iron levels. To this end, we deleted various regions within the *HAP43* gene and tested the function of the mutated proteins.

Mutants with both C- and N-terminal truncations exhibited growth defects under iron deficiency and were not able to induce the hemoglobin uptake machinery. The HapX C-terminus in *A. fumigatus* is also essential for the iron limitation response (Gsaller et al., 2014), indicating the conserved function of the C-terminus in both species. This functional similarity is further supported by the 41% amino acid sequence identity between the Hap43 and HapX C termini (Gsaller et al., 2014). The N-terminus of Hap43 is known to be necessary for the Hap/CBC complex assembly, which is required for regulation of gene expression under iron deficiency (McNabb and Pinto, 2005; Hortschansky et al., 2007; Baek et al., 2008; Hsu et al., 2013). Overall, despite the deletion of Hap43 central parts, the transcription factor remained functional, which indicates that the central part of Hap43 is not strictly required for gene regulation. However, as a minor defect was observed, the central Hap43 region might be required for proper protein folding and stability. Like in *A. fumigatus,* all single CRR deletions of Hap43 in *C. albicans* were indistinguishable from the wild type under iron deficiency conditions (Gsaller et al., 2014). Therefore, we conclude that only C- and N-termini of Hap43 are crucial for its function under iron deprivation.

As HapX was found to be important in mediating *A. fumigatus* resistance to high iron (Gsaller et al., 2014), it seemed reasonable to assume a similar function for resistance against iron toxicity in *C. albicans*. However, neither complete deletion of *HAP43* nor deletion of individual or all CRRs or a C-terminal truncation changed the growth in media with excess iron. Therefore, we conclude that Hap43 of *C. albicans*, in contrast to *A. fumigatus*, *A. nidulans,* or *Fusarium oxysporum* (Gsaller et al., 2014), is not involved in the adaptation to iron excess. Interestingly, under iron excess, *CCC1,* the vacuolar iron importer, was significantly less transcribed in *hap43*Δ/Δ compared to the *C. albicans* wild type (**Figure 6A**). Similarly to these observations in *C. albicans,* HapX in *A. fumigatus* is known to activate the transcription of *cccA,* a *CCC1* homolog, under high iron levels. However, the overexpression of *cccA* alone in *hapX*Δ largely, but not fully, restored the wild type phenotype under iron excess (Gsaller et al., 2014), showing that for full protection from iron excess, HapX must regulate additional processes, like iron acquisition and consumption, which are not regulated by Hap43 in *C. albicans*. Although Hap43 may regulate transcription of *ACO1* and *CYC1* during the shift from iron starved to replete conditions, the

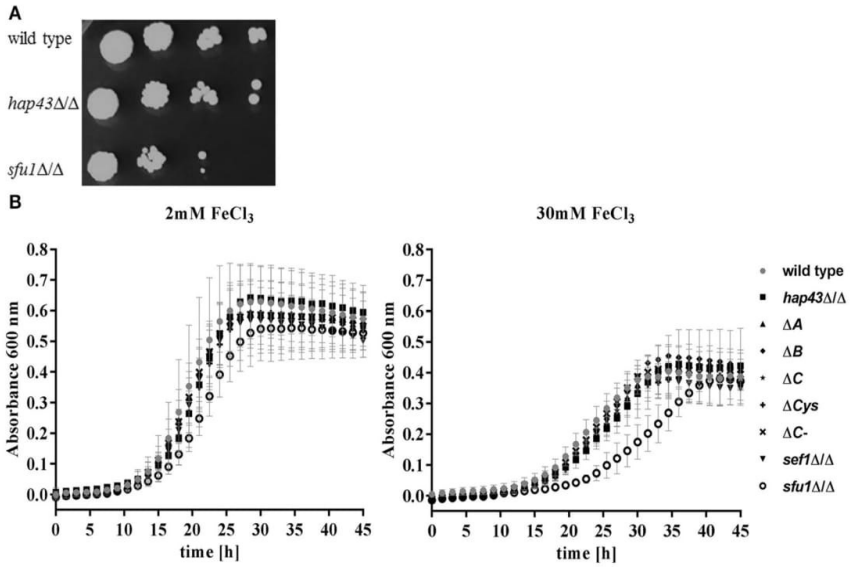

FIGURE 5 | Phenotypic profiling of the wild type and mutant strains under high iron levels. **(A)** Strains were grown on SD plates with 5 mM iron. **(B)** Strains were inoculated to OD 0.005 either in SD with 2 mM iron (iron sufficiency) or in SD with 30 mM iron (iron excess).

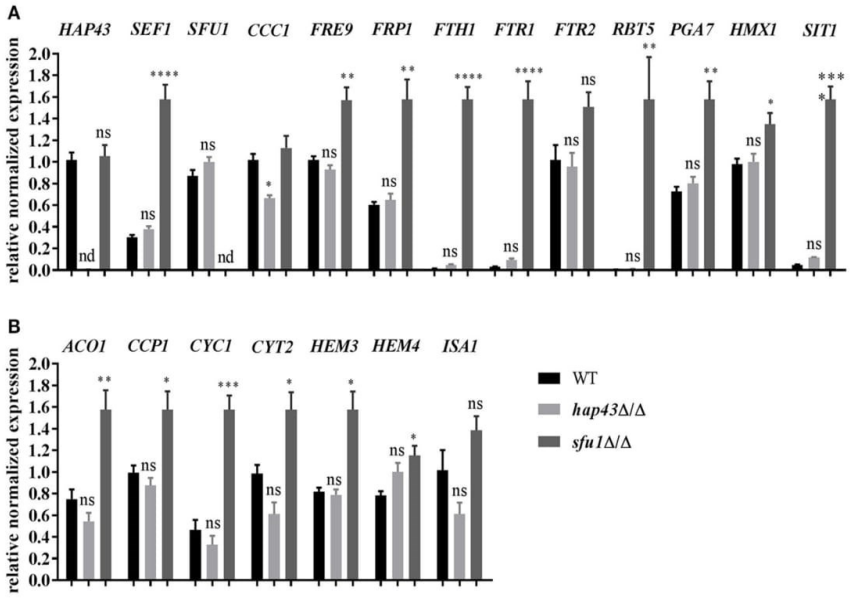

FIGURE 6 | Transcriptional profiling of iron starvation **(A)** and consumption **(B)** genes under iron excess (20 mM $FeCl_3$). The expression of iron homeostasis genes was checked *via* qRT-PCR in wild type, *hap43*Δ/Δ, and *sfu1*Δ/Δ strains. The expression was normalized to the transcript levels of *ACT1* in each strain. Asterisks indicate statistical significance compared to the wild type strain (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; ns, $p > 0.05$).

decrease in transcript levels due to Hap43 deletion was only minor compared to *A. fumigatus*. For the *hap43*Δ/Δ mutant we only observed a roughly 2-fold reduction, which is very low compared to the data shown for *A. fumigatus* (Gsaller et al., 2014), and limited to few genes even under the immediate up-shift. The difference in the expression of *C. albicans ACO1* and *CYC1* could possibly be better explained by a less severe iron shock for the *hap43*Δ/Δ mutant: Under iron limitation the wild type, but not the *hap43*Δ/Δ mutant, activates iron uptake and represses iron utilization genes (Chen et al., 2011). Upon the shift to 50 μM $FeCl_3$, both the active iron import and the repression of iron consumption likely induced a more severe short-term iron shock in the wild type than in the *hap43*Δ/Δ mutant.

Like *A. fumigatus*, *C. albicans* has another high-iron responsive factor at its disposal, Sfu1 (SreA in *Aspergillus*), which in *C. albicans* seems solely responsible to provide resistance to iron excess (Lan et al., 2004): deletion of *SFU1* resulted in an uncontrolled high expression of genes of the iron uptake machinery under iron replete conditions. Interestingly, genes for iron consuming processes were also upregulated in the *sfu1*Δ/Δ strain, which can probably be best explained as a secondary effect due to excessive influx of iron in this mutant—although even this reaction evidently did not suffice to fully detoxify the iron excess and allow for normal growth. So why are the functions of HapX and Hap43 different? While our set of deletion mutants did not reveal an immediate explanation, *in silico* analyses revealed that Hap43 and Sfu1 of *C. albicans* each contain four CRRs or single cystein, whereas in *A. fumigatus*, *A. nidulans*, and *F. oxysporum* the HapX homologs contain five CRRs and the Sfu1 homologs—three CRRs. It is tempting to speculate that these differences, *via* iron binding or protein interactions, could explain the crucial role of Sfu1, but not of Hap43, in mediating iron resistance response in *C. albicans*.

Pathogenic fungi seem to have evolved individual variants of common themes in iron homeostasis. For example, *C. albicans* has integrated an additional factor for iron acquisition, Sef1, into the established reciprocally acting pair of GATA factor (Sfu1/SreA) and CCAAT binding complex (with Hap43/HapX) (Chen et al., 2011); *C. glabrata* has combined elements of this system with the vastly different iron regulatory network of *S. cerevisiae* (Gerwien et al., 2016); and *Aspergillus* spp. and related species employ the CCAAT binding complex to counteract both iron starvation and excess. Nevertheless, *A. fumigatus* contains the SreA system, which directly interacts with HapX. Supporting other data, we have shown here that *C. albicans*, high and low iron regulation is not interwoven by Hap43 and Sfu1, but rather form a reciprocal network (Chen et al., 2011) and there is a clear discrimination between iron starvation response (Hap43) and iron excess response (Suf1). This may make sense if we look at the environments these fungi generally face: gradually shifting environments may favor bi-functional receptors for iron starvation and excess in saprophytic pathogens like *A. fumigatus*. In contrast, the gut commensal *C. albicans* likely faces rapidly changing environments, which require a decisive, yes/no type of transcriptional response without overlapping functionality of either receptor. The iron response, thus, reflects the adaptation strategies of these fungi as formed by their environments.

AUTHOR CONTRIBUTIONS

VS, SB, and BH: designed the sturdy; MB: suggested an additional experiment; VS: performed the experiments; VS and SB: evaluated and interpreted the results; VS, SB, MB, and BH: wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02388/full#supplementary-material

Figure S1 | PCR amplification of *HAP43* from cDNA. 1, 16, 17, 28 - 1 kb DNA ladder; 2 - wild type; 3 - *hap43*Δ/Δ; 4 - *hap43*Δ/Δ+*HAP43*; 5 - Δ*B*-; 6 - Δ*C*-; 7 - Δ*Cys*-; 8 - Δ*- ; 9 - Δ*N*; 10 - Δ*-*A*; 11 - Δ*-*B*; 12 - Δ*A*; 13 - Δ*B*; 14 - Δ*C*; 15 - Δ*Cys*; 18, 20, 22, 24, 26 - wild type gDNA; 19, 21, 23, 25, 27 - H_2O.

Figure S2 | Transcriptional profiling of iron consumption genes under shift from iron-limited to iron-replete conditions. The expression of iron homeostasis genes was determined *via* qRT-PCR in wild type, *hap43*Δ/Δ, Δ*A*, Δ*B*, Δ*C*, Δ*Cys*, and Δ*C*-strains. The expression was normalized to the transcript levels of *ACT1* in each strain and to the wild-type transcript levels. Asterisks indicate statistical significance compared to the wild-type strain ($*p \leq 0.05$; $**p \leq 0.01$).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table S1. **Transcriptional screening of iron-related genes.** qRT-PCR were performed after 8 h iron limitation in wild type and *hap43*Δ/Δ. All expression levels were normalized to levels of the wild type grown in YPD.

Relative normalized expression 8 h LIM

gene	wild type	SEM	*hap43*Δ/Δ	SEM
ACO1	0,17982	0,02483	6,84661	1,93165
AQY1	11,34019	1,70029	4,26883	1,39187
CCC1	0,24524	0,02686	1,84272	0,63177
CCP1	0,27232	0,03332	1,22665	0,30138
COX5	0,08038	0,01019	0,47787	0,18251
CRD2	0,03795	0,00579	0,22128	0,05295
CYC1	0,05906	0,00622	1,78412	0,45564
CYC3	0,19190	0,03236	5,73029	2,86538
CYT1	0,04298	0,00624	0,31814	0,16314
CYT2	0,27211	0,03853	2,34542	1,36418
FET3	1,02114	0,11303	1,73495	0,25015
FRE10	2,49657	0,30165	13,82030	5,35959
FRE9	1,03998	0,12083	1,92029	0,32599
FRP2	1,58378	0,23276	0,18045	0,04791
FTH1	7,21949	0,84040	7,49049	2,69045
FTH2	0,87661	0,09850	1,64671	0,24236
FTR1	5,52018	0,62207	9,55583	2,66719
FTR2	0,98994	0,10285	1,95934	0,29143
HAP43	10,17805	1,11220	0,02842	0,00411
HEM14	0,03026	0,00420	0,24671	0,18373
HEM3	0,70313	0,07215	2,62374	0,60443
HEM4	0,15466	0,03125	0,79326	0,32760
HMX1	**3,39755**	**0,38568**	**1,47290**	**0,68303**
ISA1	0,33787	0,03953	1,79983	1,56821
MMT2	0,96385	0,10019	1,04663	0,55070
MRS4	2,46817	0,29723	2,19300	1,05555
PGA10	1,24220	0,16441	1,20808	1,04092
PGA26	0,14762	0,01651	0,32651	0,05354
PGA7	**15,69319**	**2,11215**	**13,05899**	**4,34464**
RBT5	**32,33166**	**3,41346**	**13,60853**	**5,26710**
RBT51	0,84284	0,12646	0,75405	0,12754
RIM101	1,03458	0,12810	0,40241	0,24433
SEF1	1,17820	1,84794	1,49410	2,09941
SFU1	0,22548	0,02432	1,05931	0,57496
SIT1	7,99422	0,83191	4,10533	1,32237

SMF3	0,85220	0,09985	0,73210	0,14466
SOD5	0,25863	0,03908	0,04358	0,01929
YFH1	0,23650	0,02474	0,71819	0,26429

Table S2. The list of *C. albicans* strains used in this study.

Strain	Name	Name	Genotype	Reference
M1477	BWP17 +Clp30	wild type	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG + Clp30	(Citiulo, Jacobsen et al. 2012)
M2517	*hap43*Δ/Δ	*hap43*Δ/Δ	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10	this study
M2518	*hap43*Δ/Δ+*HAP43*	*hap43*Δ/Δ + *HAP43*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*	this study
M2531	*hap43*Δ/Δ+*HAP43*$_{}^{141}$*a*	Δ*-	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{141}$	this study
M2521	*hap43*Δ/Δ+*HAP43*$_{}^{373}$*c*	Δ*B*-	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{373}$	this study
M2523	*hap43*Δ/Δ+*HAP43*$_{}^{481}$*a*	Δ*C*-	ura3::imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{481}$	this study
M2528	*hap43*Δ/Δ+*HAP43*$_{}^{583}$*b*	Δ*Cys*-	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{583}$	this study
M2533	*hap43*Δ/Δ+*HAP43*$_{}^{142\text{-}634}$*a*	Δ*N*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{142\text{-}634}$	this study
M2535	*hap43*Δ/Δ+*HAP43*$_{}^{141,281\text{-}634}$*a*	Δ*-*A*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43*$_{}^{141,281\text{-}634}$	this study
M2537	*hap43*Δ/Δ+*HAP43*$_{}^{141,386\text{-}634}$*a*	Δ*-*B*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-	this study

			*HAP43_*$^{141,386-634}$	
M2539	*hap43*Δ/Δ+*HAP43_*$^{269,281-634}$	Δ*A*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43_*$^{269,281-634}$	this study
M2540	*hap43*Δ/Δ+*HAP43_*$^{373,386-634}$	Δ*B*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43_*$^{373,386-634}$	this study
M2541	*hap43*Δ/Δ+*HAP43_*$^{481,503-634}$	Δ*C*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43_*$^{481,503-634}$	this study
M2542	*hap43*Δ/Δ+*HAP43_*$^{583,585-634}$	Δ*Cys*	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *hap43*::*HIS1*/*hap43*::*ARG4* +Clp10-*HAP43_*$^{583,585-634}$	this study

Table S3. The list of primers used in this study.

Name	Sequence 5->3'	Purpose
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-HAP43
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-*HAP43_*^{141}
RPendCoil	CTGTAAGTCAAACTA CTGGTTCTCCACCTTTAATTTATTTATAGTGTT	
FPStopafterHAP43	AAGGTGGAGAACCAG TAGTTTGACTTACAGTATGAAGTATTTCCGACAC	
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-*HAP43_*^{373}
IRPCys	CTGTAAGTCAAACTA ATTGTCAACTGGAGAATCCTCATTAAAAG	
IFPCys	TCTCCAGTTGACAAT TAGTTTGACTTACAGTATGAAGTATTTCCGACAC	
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-*HAP43_*^{481}
I_IIRPCys	CTGTAAGTCAAACTAGCCACCATCAGAAGAAGTATCCTTG	
I_IIFPCys	TCTTCTGATGGTGGCTAGTTTGACTTACAGTATGAAGTATTTCCGACAC	
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-*HAP43_*^{583}
I_II_IIIPRCys	CTGTAAGTCAAACTATGGAATGAAAATACCACTATTGCTCAG	
I_II_IIIFPCys	GGTATTTTCATTCCATAGTTTGACTTACAGTATGAAGTATTTCCGACAC	
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-*HAP43_*$^{142-634}$
RPendRromStartCod	GTTTTTAACCAAAAACATGTTGTTCAAATTGAAATTCTAATTATTAT	
FPrAcoiledcoilReg	AATTTGAACAACATGTTTTTGGTTAAAAACTTGGAACAGTTAAAAGGT	
RPDHap43ov	GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	
FPUHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG	Plasmid generation Clp10-
RPendCII_III_IV	TAACCCCACAGATTCCTGGTTCTCCACCTTTAATTTATTTATAGTGTT	
AfterCysI	AAGGTGGAGAACCAGGAATCTGTGGGGTTAAAGGAGCCCAC	

RPDHap43ov	gggaacaaaagctggGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	*HAP43_*$^{141,281-634}$
FPUHap43ov RPendCIII_IV AftCysII RPDHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG TTTAGCAGCTTCACGCTGGTTCTCCACCTTTAATTTATTTATAGTGTT AAGGTGGAGAACCAGCGTGAAGCTGCTAAAGAAGCAGCAAA GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	Plasmid generation CIp10-*HAP43_*$^{141,386-634}$
FPUHap43ov RPBCysIov FPACysIov RPDHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG TAACCCCACAGATTCATCAAAGTTAGCTACATCAGCGGCG GTAGCTAACTTTGATGAATCTGTGGGGTTAAAGGAGCCC GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	Plasmid generation CIp10-*HAP43_*$^{269,281-634}$
FPUHap43ov RPBCysIIov FPACysIIov RPDHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG TTTAGCAGCTTCACGATTGTCAACTGGAGAATCCTCATTAAAAG TCTCCAGTTGACAATCGTGAAGCTGCTAAAGAAGCAGC GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	Plasmid generation CIp10-*HAP43_*$^{373,386-634}$
FPUHap43ov RPBCysIIIov FPACysIIIov RPDHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG ACTGGCAACAGTAGTGCCACCATCAGAAGAAGTATCCTTG TCTTCTGATGGTGGCACTACTGTTGCCAGTCGAAGTACAAAATC GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	Plasmid generation CIp10-*HAP43_*$^{481,503-634}$
FPUHap43ov RPBCysIIIIov FPACysIIIIov RPDHap43ov	TCGATACCGTCGACCTACATACAGCTAAAAGTGTGTCGTAGTTTATTGG CTTATAAGCATCTGCTGGAATGAAAATACCACTATTGCTCAG GGTATTTTCATTCCAGCAGATGCTTATAAGACTTTGTCTCGTCAT GGGAACAAAAGCTGGGTTTGTTTGTTTGTTTGTTTGAGATTTTTATAGTAA	Plasmid generation CIp10-*HAP43_*$^{583,585-634}$
URA-F2 RPF-1	GGAGTTGGATTAGATGATAAAGGTGATGG GAGCAGTGTACACACACACATCTTG	Conrormation of plasmid integration
RPF-2 M13R1	CGCCAAAGAGTTTCCCCTATTATC AGCGGATAACAATTTCACACAGGA	Conrormation of plasmid integration
Fhap Rhap 4_R 5_R 6_R 7_R 8_F	TTGCAATAGCAGGATCACCA TCGTAACACGTTGGCTACTGA CAGTTTCAGTTTGGGGTTGAG TCTTGGAAGCTGGCAATACA GCTCAGAACCGACGAAGAGT GCCATTTCCTTTATGTCCCTTT AGGAGCCCACAAAAGACAAA	*HAP43* expression check from cDNA samples
EF1B-F EF1B-R	AGTCATTGAACGAATTCTTGGCTG TCTTCATCAACTTCATCATCAGAACC	gDNA contamination
ACT1-F ACT1-R rtFCCC1 rtRCCC1 rtFRBT5 rtRRBT5 rtFHAP43 rtRHAP43 rtFSIT1	TCAGACCAGCTGATTTAGGTTTG GTGAACAATGGATGGACCAG TGGGTGAAGGTTGTCCAAAT CAAGAACACCAATCCCCAAG CTGCTGAAAGTTCTGCACCA GCTTCAACGGAAACAGAAGC TGAACAACCCTCACCAATCA TCTTGGAAGCTGGCAATACA TGCTATGTGGATGGTTGCAT	qRT-PCR

rtRSIT1	CAATGCCGATGAAATCACAG	
rtFHMX1	AATTTGCCCTTGCTTTGAGA	
rtRHMX1	GTCTTGCTCGGCTTTACCAG	
rtFFRP1	GGGTGGTGCATTTACATTCC	
rtRFRP1	TCCAGCAAACTGTTTCAACG	
rtFFRE9	AAGAAGGCGATGGTACGAAA	
rtRFRE9	CAATGCCGATGAAATCACAG	
rtFPGA7	CAGGATGTTTCTGCGTGATG	
rtRPGA7	GCCAAGAATCTGGCACTAGC	
rtFSEF1	GTTGCTCCATTTGCATCTCA	
rtRSEF1	CAAACCAACCCAAAAAGTCG	
rtFSFU1	ATCAAACAGCACCGCTCTCT	
rtRSFU1	GGTCCGGGTCCATTAGGTAT	
rtFFTR1	GCCGGTATCGTTGTTGGTGC	
rtRFTR1	GTGTTGGTTTCGAAATACCAAATACCTC	
rtFFTR2	TGTGGTCTTGCAGTGGGTG	
rtRFTR2	GAGGTGTCTGGTTCTTTGAGAGTTA	
rtFFTH1	GCTGGTTTGTTCTCCAGAGG	
rtRFTH1	ACCGTGACCAGTCTCACTCA	

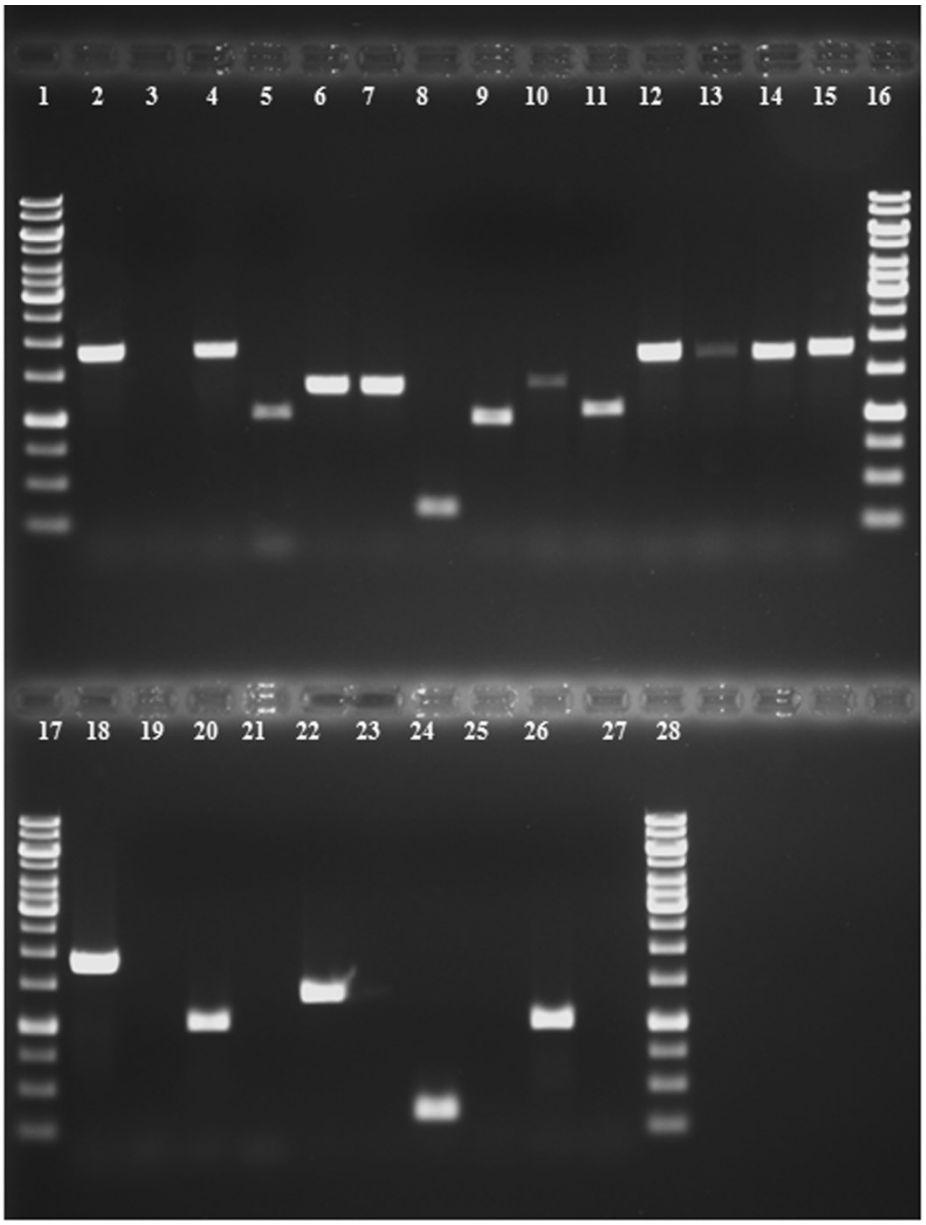

Figure S1. PCR amplification of *HAP43* from cDNA. 1, 16, 17, 28 - 1 kb DNA ladder; 2 - wild type; 3 - *hap43*Δ/Δ; 4 - *hap43*Δ/Δ+*HAP43*; 5 - Δ*B*-; 6 - Δ*C*-; 7 - Δ*Cys*-; 8 - Δ*- ; 9 - Δ*N*; 10 - Δ*-*A*; 11 - Δ*-*B*; 12 - Δ*A*; 13 - Δ*B*; 14 - Δ*C*; 15 - Δ*Cys*; 18, 20, 22, 24, 26 - wild type gDNA; 19, 21, 23, 25, 27 - H_2O.

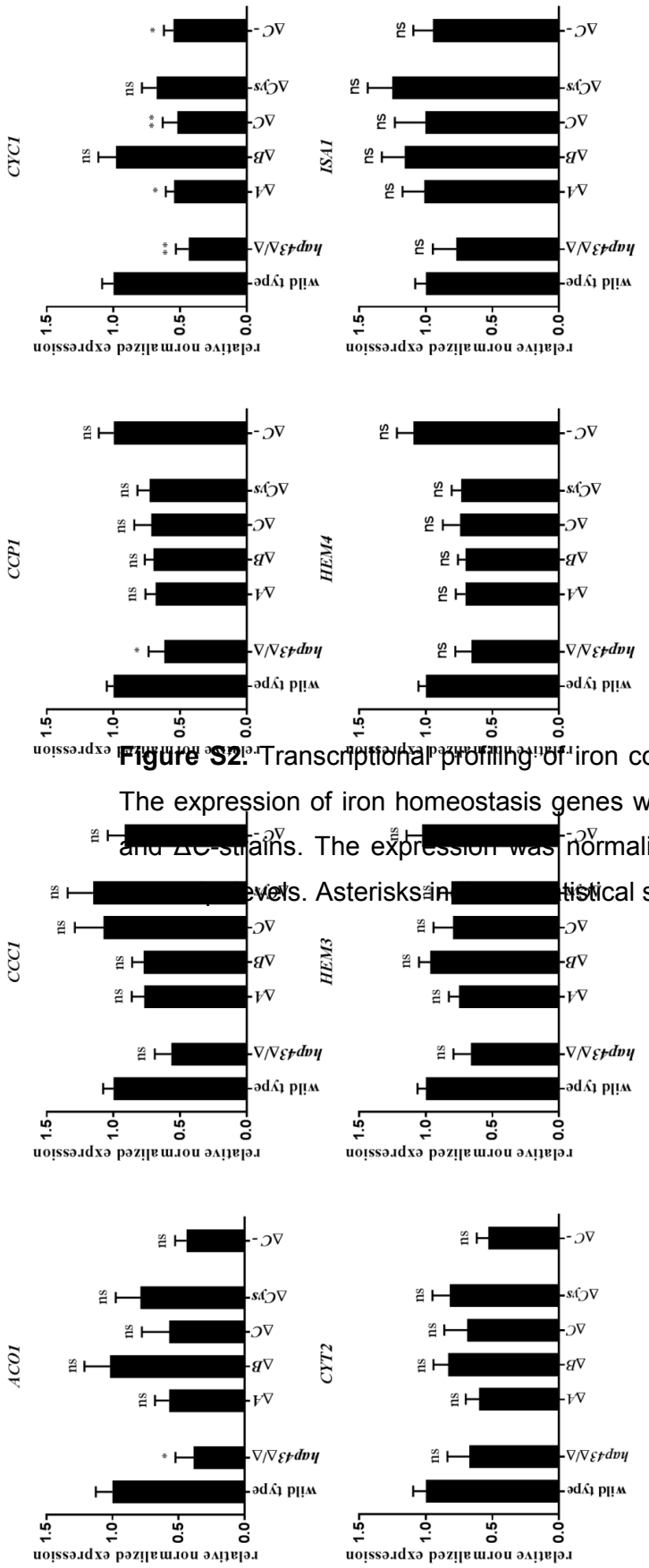

Figure S2. Transcriptional profiling of iron consumption genes under shift from iron-lim

The expression of iron homeostasis genes was determined *via* qRT-PCR in wild type, *h* and ΔC-strains. The expression was normalized to the transcript levels of *ACT1* in ea levels. Asterisks in[illegible]tistical significance compared to the wild-type strain

4.2. Manuscript II: Crawford *et al*., *PLOS Pathogens*, 2018

Biphasic zinc compartmentalisation in a human fungal pathogen

Aaron C. Crawford, Laura E. Lehtovirta-Morley, Omran Alamir, Maria J. Niemiec, Bader Alawfi, Mohammad Alsarraf, **Volha Skrahina**, Anna C. B. P. Costa, Andrew Anderson, Sujan Yellagunda, Elizabeth R. Ballou, Bernhard Hube, Constantin F. Urban, Duncan Wilson

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Summary:

The host nutritional immunity applies both zinc limitation and overload strategies in order to inhibit microbial growth. *Candida albicans* colonizes the gut in healthy individuals, however it is also able to disseminate and cause severe infections in immunocompromised patients. Thus, the fungus has evolved to tolerate diverse environments within the host, including those that vary in zinc availability. The zinc transport machinery in response to diverse zinc levels was characterized in *C. albicans*. Under external zinc limitation the uptake of zinc is performed *via* plasma membrane zinc importers, the function of which is pH dependent: Zrt2 assimilates zinc at acidic pH and Zrt1 at alkaline pH. The pH regulated zinc uptake system in *C. albicans* is similar to the one that has been described for *Aspergillus fumigatus*. Furthermore, in *C. albicans*, Zrt2 is indispensable for virulence and hence, mice infected with the *zrt2*Δ/Δ mutant, possess a decreased fungal burden in kidney when compared to the wild type infected mice. Under extracellular high zinc levels, zinc is imported *via* Zrc1 into zincosomes, which is in contrast to the situation in *Saccharomyces cerevisiae*, where zinc is rapidly compartmentalized by Zrc1 into vacuoles. In response to inflammation, the host causes zincaemia *via* the transport of a labile zinc pool into the liver. The presence of Zrc1 in *C. albicans* was found to be essential for resistance to high zinc levels and liver colonization. As a consequence, both zinc uptake and storage processes are critical for *C. albicans* survival and virulence.

Own Contribution:

Volha Skrahina generated the *zrt1*Δ/Δ*zrt2*Δ/Δ mutant and *zrt1*Δ/Δ*zrt2*Δ/Δ+*ZRT1/ZRT2* revertant strains. These strains were required for phenotypic profiling and assimilation assays, the results of these experiments are represented in three manuscript figures. These data are critical for the confirmation of the hypotheses about the pH dependent regulation and metal specificity of zinc transporters in *C. albicans*. Volha Skrahina

interpreted results and suggested experiments for the revision of the manuscript. The other authors designed the project, carried out *in vitro* and *in vivo* experiments, interpreted results, prepared figures, wrote and revised the manuscript.

Estimated authors' contributions:

Aaron C. Crawford	20%
Laura E. Lehtovirta-Morley	10%
Omran Alamir	10%
Maria J. Niemiec	6%
Bader Alawfi	6%
Mohammad Alsarraf	6%
Volha Skrahina	**6%**
Anna C. B. P. Costa	2%
Andrew Anderson	2%
Sujan Yellagunda	1%
Elizabeth R. Ballou	2%
Bernhard Hube	2%
Constantin F. Urban	2%
Duncan Wilson	25%

Prof. Bernhard Hube

Manuscript II

Biphasic zinc compartmentalisation in a human fungal pathogen

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Abstract

Nutritional immunity describes the host-driven manipulation of essential micronutrients, including iron, zinc and manganese. To withstand nutritional immunity and proliferate within their hosts, pathogenic microbes must express efficient micronutrient uptake and homeostatic systems. Here we have elucidated the pathway of cellular zinc assimilation in the major human fungal pathogen *Candida albicans*. Bioinformatics analysis identified nine putative zinc transporters: four cytoplasmic-import Zip proteins (Zrt1, Zrt2, Zrt3 and orf19.5428) and five cytoplasmic-export ZnT proteins (orf19.1536/Zrc1, orf19.3874, orf19.3769, orf19.3132 and orf19.52). Only Zrt1 and Zrt2 are predicted to localise to the plasma membrane and here we demonstrate that Zrt2 is essential for *C. albicans* zinc uptake and growth at acidic pH. In contrast, *ZRT1* expression was found to be highly pH-dependent and could support growth of the *ZRT2*-null strain at pH 7 and above. This regulatory paradigm is analogous to the

distantly related pathogenic mould, *Aspergillus fumigatus*, suggesting that pH-adaptation of zinc transport may be conserved in fungi and we propose that environmental pH has shaped the evolution of zinc import systems in fungi. Deletion of *C. albicans ZRT2* reduced fungal burden in wild type, but not in mice lacking the zinc-chelating antimicrobial protein calprotectin. Inhibition of *zrt2*Δ growth by neutrophil extracellular traps was calprotectin-dependent. This suggests that, within the kidney, *C. albicans* growth is determined by pathogen-Zrt2 and host-calprotectin. As well as serving as an essential micronutrient, zinc can also be highly toxic and we show that *C. albicans* deals with this potential threat by rapidly compartmentalising zinc within vesicular stores called zincosomes. In order to understand mechanistically how this process occurs, we created deletion mutants of all five ZnT-type transporters in *C. albicans*. Here we show that, unlike in *Saccharomyces cerevisiae*, *C. albicans* Zrc1 mediates zinc tolerance *via* zincosomal zinc compartmentalisation. This novel transporter was also essential for virulence and liver colonisation *in vivo*. In summary, we show that zinc homeostasis in a major human fungal pathogen is a multi-stage process initiated by Zrt1/Zrt2-cellular import, followed by Zrc1-dependent intracellular compartmentalisation.

Author's summary

All living organisms must secure certain trace metals such as iron and zinc in their diets. For the microbes that infect us, the source of these micronutrients is the tissues of their host. However, mammals have developed sophisticated mechanisms to manipulate microbial access to trace metals – a process called nutritional immunity. Therefore, successful pathogenic microorganisms must have evolved mechanisms to counteract nutritional immunity and acquire micronutrients in order to grow within their hosts and cause disease. This struggle for micronutrients represents a key host-pathogen battleground. In this study we demonstrate how the major human fungal pathogen, *Candida albicans*, acquires and stores zinc from its environment. We find that the mechanistic basis of zinc uptake is highly dependent on the acidity of the surrounding environment. Interestingly, this pH-dependence appears conserved in the fungal kingdom and we propose a potential framework for the evolution of zinc uptake in extant fungal species. Moreover, following cellular assimilation, *C. albicans* shuttles this potentially toxic transition metal into subcellular compartments called zincosomes. We also show that both zinc uptake and compartmentalisation are critical for *C. albicans* growth, both under laboratory conditions and in experimental models of invasive candidiasis.

Introduction

Certain trace metals such as iron and zinc (collectively termed micronutrients) are essential for cellular life, and at least a third of all proteins interact with a metal cofactor (Waldron, Rutherford et al. 2009). Zinc is particularly important for eukaryotes as around 9% of their proteomes require this metal for function (Andreini, Bertini et al. 2009). However, these essential metals can also be highly toxic to cells, and precise metal ion homeostasis is critical for survival. Pathogenic microorganisms face a complicated relationship with micronutrients as the mammalian host uses both high antimicrobial concentrations of metals, as well as metal sequestration to kill microbes or inhibit their growth. Collectively, these processes are known as *nutritional immunity* (4). The “battle for iron” is an established paradigm in host-pathogen interactions (Skaar 2010) and, more recently, important roles for manganese, copper and zinc have emerged within the framework of nutritional immunity (Hood and Skaar 2012). Zinc in particular represents a double-edged sword for potentially invasive species. Botella *et al*. established that phagocytosed *Mycobacterium tuberculosis* cells experience acute zinc toxicity within macrophages, and that intracellular survival is reliant on heavy metal efflux P-type-ATPase activity (Botella, Peyron et al. 2011). In other host niches, zinc availability is extremely limited due to systemic zincaemia or locally produced zinc-chelating agents such as calprotectin. In these environments, efficient zinc uptake is crucial for pathogenicity, and a number of recent studies have demonstrated the importance of the *znuABC* high affinity zinc importer for bacterial virulence (Hood and Skaar 2012, Becker and Skaar 2014).

Fungi do not appear to encode ABC transport systems for zinc acquisition. Instead, eukaryotic zinc transport can be mediated by members of two protein families: the Zip and ZnT transporters, which transport zinc into and out of the cytoplasm, respectively (Eide 2006). In the model yeast *S. cerevisiae*, Zip family members have been shown to assimilate zinc from the environment or to export zinc from intracellular organelles such as the vacuole (Zhao and Eide 1996, Zhao and Eide 1996, MacDiarmid, Gaither et al. 2000). In contrast, ZnT proteins play roles in organellar zinc accumulation. In *S. cerevisiae*, the major target for excess zinc is the vacuole (Simm, Lahner et al. 2007), as well as small vesicular zinc storage compartments called zincosomes (Devirgiliis, Murgia et al. 2004). Whilst vacuolar zinc import is mediated by the ZnT-type transporter Zrc1 (MacDiarmid, Milanick et al. 2002), the mechanism of fungal intracellular zincosomal zinc compartmentalisation is not known (Devirgiliis, Murgia et al. 2004).

Predicted plasma membrane Zip transporters have now been characterised in the major human fungal pathogens *Aspergillus fumigatus*, *H. capsulatum*,

C. neoformans and *Cryptococcus gattii* (Amich, Vicentefranqueira et al. 2014, Schneider Rde, Diehl et al. 2015, Dade, DuBois et al. 2016, Do, Hu et al. 2016), and Zip transporter mutants for all four species exhibit attenuated virulence, underscoring the importance of zinc uptake for fungal, as well as bacterial pathogenicity.

Candida albicans is a normal commensal member of the human gastrointestinal microbiota and other mucosal surfaces, a common cause of mucosal infections, and a serious invasive pathogen in certain patient groups (Brown, Denning et al. 2012). In fact, invasive candidiasis, predominantly caused by *C. albicans*, affects more than a quarter of a million individuals each year and is responsible for at least 50,000 deaths annually (Kullberg and Arendrup 2015). We have previously shown that this fungus can scavenge zinc *via* the secreted protein Pra1 and that this "zincophore" system is important for host cell damage in tissue culture infection models (Citiulo, Jacobsen et al. 2012). However, a *pra1*Δ mutant is hyper-virulent in a mouse model of infection as it also serves as a ligand for neutrophil alphaMbeta2 (Soloviev, Fonzi et al. 2007, Soloviev, Jawhara et al. 2011).

In this study we have functionally dissected zinc transport in *C. albicans*. We identified nine putative zinc transporters including two predicted plasma membrane Zip proteins, Zrt1 and Zrt2, as well as five ZnT proteins. Regulatory and functional analysis demonstrates that pH-dependent adaptation to zinc limitation may be conserved in fungi, but that distinct transporter subclasses differentially contribute to growth *in vivo* for different human pathogenic species. Moreover, for the first time, we define a molecular mechanism of zincosomal zinc accumulation in a human fungal pathogen.

Results and Discussion

Identification of zinc importers in Candida albicans.

Zinc transport in eukaryotes can be mediated by members of the Zip and ZnT protein families, which transport their substrate to or from the cytoplasm, respectively (Eide 2006). In order to determine how *C. albicans* acquires zinc from its environment, we first focussed on Zip transporters. Using the FungiDB (Stajich, Harris et al. 2012) InterPro domain-finder (PFAM: PF02535; http://fungidb.org/fungidb/) we identified four Zip-type *C. albicans* proteins. Only Zrt1 and Zrt2 are predicted plasma membrane transporters. In contrast, Zrt3 and orf19.5428 share similarity with *S. cerevisiae* Zrt3 (vacuolar zinc) and Atx2 (Golgi manganese) transporters. We had previously generated a *C. albicans zrt1*Δ mutant as part of our efforts to characterise the fungal zincophore, Pra1 (Citiulo, Jacobsen et al. 2012). In this previous study we found that Zrt1 was essential for the reassociation of soluble Pra1 to the fungal cell surface,

indicating that Zrt1 is likely cell surface-localised. *ZRT2*, on the other hand, was (at time of writing) annotated in the *Candida* Genome Database (www.candidagenome.org/) (Inglis, Arnaud et al. 2012) as a possibly essential gene (Aaron Mitchell, personal communication to the CGD). Indeed, our initial attempts to delete the second allele of *ZRT2* were unsuccessful (the first 104 second-round clones retained their second allele of *ZRT2*). Supplementation of the transformation selection medium with 1 mM $ZnSO_4$ permitted the successful isolation of a *C. albicans zrt2*Δ homozygous mutant, suggesting that *ZRT2* is conditionally essential.

Zrt1 and Zrt2 - pH dependent zinc acquisition.

Subsequent attempts to culture *C. albicans zrt2*Δ in SD (YNB + glucose) medium (the minimal yeast growth medium, routinely used for the selection of transformants) failed, indicating that *ZRT2* is indeed essential for growth under this laboratory condition. Consistent with conditional essentiality, growth of *zrt2*Δ was restored to wild type levels *via* zinc supplementation or by genetic complementation with a single copy of *ZRT2* (**Figure 1A**). Deletion of *ZRT1* did not impact growth in SD medium. We also tested growth in liquid and agar hyphae-inducing medium and under biofilm conditions, but observed no difference between wild type and *zrt1*Δ or *zrt2*Δ strains (**Supplementary Figure S1 and S2**).

In the pathogenic mould *A. fumigatus*, Zrt1 and Zrt2 orthologues (ZrfC and ZrfB) are required for zinc uptake at neutral/alkaline and acidic pH, respectively (Vicentefranqueira, Moreno et al. 2005, Amich, Leal et al. 2009, Amich, Vicentefranqueira et al. 2010). Although pH-dependent zinc transport has not been reported in the more closely related yeast, *S. cerevisiae*, a previous study has indicated that *C. albicans ZRT1* and *ZRT2* are also pH-regulated (Bensen, Martin et al. 2004). As SD minimal medium has a native pH of ~4.8, we tested the effect of neutralising the growth medium. Buffering the medium to pH 7.4 restored growth of *zrt2*Δ, and had no adverse effect on *zrt1*Δ, which again grew to wild type levels (**Figure 1A**). Similar pH dependent growth patterns and zinc rescue effects were observed in synthetic limited zinc medium (**Figure S3**).

As *C. albicans* encodes only two predicted plasma membrane zinc importers, these data indicated that, in laboratory medium, Zrt1 can support growth at neutral-alkaline pH, whilst Zrt2 is essential for growth at acidic pH. Based on these growth patterns, we hypothesised that *ZRT1* is specifically expressed at neutral/alkaline pH, whilst *ZRT2* expression is pH-independent. We note that this regulatory and functional model aligns more closely with that of the pathogenic mould *A. fumigatus*

(Vicentefranqueira, Moreno et al. 2005, Amich, Leal et al. 2009, Amich, Vicentefranqueira et al. 2010).

To test this hypothesis we constructed *C. albicans* reporter strains with GFP (Cormack, Bertram et al. 1997) expression driven from either the *ZRT1* or *ZRT2* promoters. **Figure 1B** shows the expression profiles of P_{ZRT1} and P_{ZRT2} in low-zinc medium at a range of environmental pH values. GFP fluorescence driven by P_{ZRT1} activity was low at pH 4.6. However, as the media was neutralised, fluorescence increased. Expression was 40-fold higher at pH 7.5 than at pH 4.6. At pH 6.5 and above, P_{ZRT1}-GFP expression was significantly higher than at pH 4.6.

In contrast, P_{ZRT2}-GFP expression was not as strongly affected by the pH of the surrounding media, with expression at pH 4.6 being only 2-fold higher than at pH 6.5. These data are in agreement with the previous study of Bensen *et al*. who reported alkaline- and acidic- induction of *ZRT1* and *ZRT2*, respectively (Bensen, Martin et al. 2004). However, from our own observations, we conclude that expression of *ZRT1* is more strongly influenced by environmental pH than *ZRT2*.

These expression data support our hypothesis that Zrt2 is essential in acidic environments, whilst either Zrt1 or Zrt2 can support growth at neutral pH. To test this directly we created a *zrt1*Δ/*zrt2*Δ double mutant and performed more detailed growth kinetics analysis. **Figure 1C** shows that *zrt2*Δ again grew at neutral, but not acidic pH, whilst *zrt1*Δ/*zrt2*Δ failed to grow at both pH values. Growth was fully restored in the revertant strain (**Figure 1C**).

The above growth and expression assays indicated that Zrt2 is the dominant cellular zinc transporter in *C. albicans* and the only functional importer at acidic pH. To test this, wild type, *zrt2*Δ and *zrt2*Δ+*ZRT2* were cultured in low zinc medium (SD zinc-dropout, acidic), provided with 25 μM Zn^{++} and zinc uptake from the medium measured. Wild type *C. albicans* sequestered all measurable zinc within 60 minutes. Zinc uptake was virtually abolished in the *zrt2*Δ mutant and *ZRT2* complementation restored uptake to 68% (**Figure 2A**). Therefore Zrt2 is essential for zinc acquisition by yeast cells in SD minimal medium.

We next assessed the relative impact of Zrt1 and Zrt2 on zinc uptake at neutral pH in RPMI medium (pH 8.2) at 37°C in tissue culture plates. Under these conditions the wild type took up 74% of zinc from the medium by 180 min (**Figure 2B**). In line with our observations that *ZRT1* and *ZRT2* are expressed at neutral pH, both *zrt1*Δ and *zrt2*Δ mutants acquired zinc from the medium, but this was reduced by approximately 50% compared to the wild type. Simultaneous deletion of both *ZRT1* and *ZRT2* abolished zinc uptake. Respective complementation with *ZRT1* and/or *ZRT2* increased

zinc uptake to 55-63%. Therefore, both Zrt1 and Zrt2 contribute to zinc acquisition in RPMI.

In summary, Zrt2 is the major zinc importer in *C. albicans* whilst Zrt1 can support zinc uptake and growth specifically at neutral/alkaline pH.

Both transporters are members of the Zip (Zrt/Irt protein) family, which also include iron transporters. Therefore, to assess the metal specificity of *ZRT1* and *ZRT2* regulation, we tested their expression in response to zinc and three other physiologically relevant trace metals - iron, manganese and copper. The reporter strains were incubated in low zinc media, buffered to pH 5 or to pH 7.5, and supplemented with zinc, iron, manganese or copper at 100 µM. At pH 5, P_{ZRT1} activity was again very low and supplementation with the different metals had no appreciable effect on expression (**Figure 3A**). At pH 7.5, P_{ZRT1} was 13.4-fold induced compared to pH 5 (**Figure 3A *vs.* 3B**). The addition of zinc to the medium resulted in 40-fold repression of P_{ZRT1} whilst iron, manganese and copper supplementation had no effect (**Figure 3B**).

P_{ZRT2} was again active in both acidic and neutral/alkaline media. At pH 5 and pH 7.5, zinc supplementation resulted in 6.5- and 3.2- fold repression, respectively. Supplementation with iron, manganese or copper had no effect (**Figure 3C & 3D**). From these data, we conclude that the metallo-regulation of *ZRT1* and *ZRT2* is zinc-specific in *C. albicans*.

In order to functionally assess metal specificity, wild type, *zrt2*Δ and *zrt1*Δ/*zrt2* cells were again cultured in minimal media, supplemented with zinc, iron, manganese, or copper. **Figure 4** shows that zinc, but not iron, manganese or copper supplementation restored growth, indicating that the growth defect of these mutants is due to an inability to acquire zinc in minimal media.

From these *in vitro* assays, it would appear that zinc transport in *C. albicans* is actually more similar to *A. fumigatus* than to *S. cerevisiae*. Baker's yeast encodes two plasma membrane importers: the high affinity Zrt1 and low affinity Zrt2, neither of which are known to be subject to pH-regulation (Zhao and Eide 1996, Zhao and Eide 1996). In contrast, *A. fumigatus* encodes three zinc importers: ZrfA and ZrfB, which are expressed in acidic environments, and ZrfC, which is expressed at neutral/alkaline (Vicentefranqueira, Moreno et al. 2005, Amich, Vicentefranqueira et al. 2010). As *A. fumigatus* ZrfB and ZrfC are respective orthologues of *C. albicans* Zrt2 and Zrt1 (Wilson 2015), this suggests that zinc transporter pH-dependence may be conserved in multiple fungal species. In (Wilson 2015) and in supplementary data **Figure S4** we

propose an evolutionary framework of how pH adaptation may have shaped the evolution of fungal zinc transporters.

***Zinc uptake during invasive candidiasis*.**

The role of zinc uptake in *C. albicans* virulence remains largely unexplored. Here we used a murine model of disseminated candidiasis to directly assess the role of Zrt1 and Zrt2 in *C. albicans* fitness *in vivo*. Mice were infected intravenously and kidney fungal burden assessed at day one and day three post-infection. **Figure 5A** shows that by day one post-infection, all strains exhibited similar levels of kidney fungal burden, indicating that neither Zrt1 nor Zrt2 are required for initial kidney colonisation.

However, by day three post-infection, *C. albicans* wild type kidney fungal burden had increased significantly by 6.5-fold (P = 0.034), indicating that cells had proliferated in this organ. In contrast, deletion of *ZRT2* precluded an increase in kidney fungal burden between day one and day three post-infection (P = 0.597). Complementation of *zrt2*Δ with a single copy of *ZRT2* restored kidney colonisation at day three (4.5-fold higher than at day one, P = 0.004). In contrast, deletion of *ZRT1* did not inhibit fungal proliferation in the kidney. These data indicate that Zrt1 and Zrt2 are dispensable for initial kidney colonisation (day one post-infection) and that Zrt2 is important for systemic candidiasis at later stages. These data are in agreement with previous studies. Xu and co-workers identified a transcription factor, Sut1, which governs the expression of zinc assimilation genes during invasive candidiasis (Xu, Solis et al. 2015). Deletion of *SUT1* attenuated *C. albicans* virulence, however, *sut1*Δ virulence was restored to wild type levels *via ZRT2* overexpression, indicating that defective *in vivo* expression of *ZRT2* was responsible for the attenuated virulence of *sut1*Δ (Xu, Solis et al. 2015). Several other studies have analysed the *C. albicans* transcriptome during kidney colonisation. Walker and co-workers reported that only two genes were transcriptionally upregulated during both rabbit (Andes, Lepak et al. 2005) and mouse (Walker, Maccallum et al. 2009) kidney colonisation: *ADR1* and *ZRT2*. *ZRT2* is also upregulated during *in vitro* incubation with macrophages (Lorenz, Bender et al. 2004). Combined with the *zrt2*Δ *in vivo* growth defect reported here, these expression studies suggest an important role for Zrt2 in zinc uptake during invasive candidiasis.

We next addressed the role of host-driven nutritional immunity on fungal growth *in vivo*. Calprotectin plays a key role in mediating zinc nutritional immunity. Calprotectin is a heterodimeric protein composed of S100A8 and S100A9 subunits which has potent antifungal activity *via* zinc sequestration (Urban, Ermert et al. 2009, Besold, Gilston et

al. 2017). Calprotectin expression in *C. albicans*-infected murine kidney tissue has been reported to be upregulated between day one and day three post-infection in two independent studies (Hebecker, Vlaic et al. 2016, Besold, Gilston et al. 2017). As Zrt2 is important for growth under zinc limitation *in vitro* and exhibited impaired growth *in vivo*, we examined the impact of calprotectin on *C. albicans* kidney colonisation. Surprisingly, all five tested *C. albicans* strains exhibited lower kidney fungal burdens in calprotectin-deficient mice than in wild type animals at both day one and day three post-infection (**Figure 5B**). At day three post-infection, the fungal burden of calprotectin-deficient mouse kidneys infected with wild type *C. albicans* was significantly lower ($p < 0.05$) than wild type mice infected with the same strain. This was unexpected, as calprotectin-deficient mice have been previously shown to succumb earlier to *C. albicans* infections (Urban, Ermert et al. 2009), however a recent study has also reported lower kidney fungal burden in calprotectin deficient mice compared to wild type (Besold, Gilston et al. 2017). In addition to its anti-fungal activity *via* zinc sequestration, calprotectin plays additional roles in immunity. Indeed, as well as its role in nutritional immunity, calprotectin has been implicated as an inflammatory mediator and has been shown to exacerbate disease in other models of candidiasis (Yano, Noverr et al. 2012). These additional immune properties may explain the decreased fungal burden observed in calprotectin-deficient mice. Nevertheless, in calprotectin-deficient mice, the *zrt2*Δ mutant did not exhibit a notable difference in kidney colonisation compared to wild type *C. albicans*. This indicates that, in the absence of a host calprotectin response, fungal Zrt2 is dispensable for kidney colonisation by *C. albicans*.

Calprotectin constitutes around half the cytoplasmic protein content of neutrophils and is a major component of neutrophil extracellular traps (NETs), from which it elicits its antifungal activity *via* zinc sequestration (Urban, Ermert et al. 2009, Niemiec, De Samber et al. 2015). In order to explore the host-pathogen relationship between pathogen Zrt2 and host calprotectin in greater detail, we next compared the antifungal properties of wild type and calprotectin-deficient NETs.

Calprotectin-decoration of NETs and associated antifungal activity *via* zinc sequestration has been well defined (Urban, Ermert et al. 2009). In line with this, S100A9-/- NETs exhibited highly attenuated antifungal activity compared to NETs from wild type neutrophils (**Supplementary Figure S6**). Deletion of *ZRT2* rendered *C. albicans* sensitive to NET antifungal activity in a calprotectin-dependent manner (**Figure 6**), suggesting a role for Zrt2 in growth in the presence of calprotectin$^+$ NETs. In summary, host (calprotectin) and pathogen (Zrt2) factors appear to define the struggle for zinc during *C. albicans* infection: Zrt2 is the major zinc transporter of this important

fungal pathogen and is essential for growth in the presence of calprotectin *in vivo* and *ex vivo*.

From this study, and work from the groups of Mitchell, Calera, Deepe, Staats and Jung, it is becoming increasing clear that zinc acquisition plays a critical role in fungal pathogenesis, as perturbation of zinc transporter function in *C. albicans*, *A. fumigatus*, *H. capsulatum*, *C. neoformans* and *C. gattii* attenuates virulence in all five organisms tested thus far (Amich, Vicentefranqueira et al. 2014, Schneider Rde, Diehl et al. 2015, Dade, DuBois et al. 2016, Do, Hu et al. 2016). Moreover, deletion of the master regulator gene of zinc homeostasis in fungi, *ZAP1*, also attenuates virulence in *A. fumigatus*, *C. gattii* and *C. dubliniensis* and decreases *in vivo* fitness in *C. albicans* (Moreno, Ibrahim-Granet et al. 2007, Noble, French et al. 2010, Schneider Rde, Fogaca Nde et al. 2012, Bottcher, Palige et al. 2015). In supplementary information **Figure S5** we discuss how different zinc uptake genes are differentially required for virulence in the major fungal pathogens of humans.

The role of intracellular compartmentalisation in adaptation to environmental zinc

We next sought to address how the fungal cell deals with zinc following its internalisation. This is an important issue because, as well as serving as an essential micronutrient, zinc can be highly toxic to cells. In order to assess the dynamics of intracellular zinc compartmentalisation, we utilised zinquin. Zinquin is a zinc-specific fluorescent probe which accumulates in storage vesicles known as zincosomes and fluoresces upon zinc binding (Wellenreuther, Cianci et al. 2009).

Zinc-depleted cells, prepared by growing the cells overnight in low zinc medium, were pulsed with 25 μM zinc, washed and fixed at five minute intervals and stained with zinquin. **Figure 7** shows that even with immediate washing and fixation, *C. albicans* already stained positive with zinquin, indicating that zincosomal zinc compartmentalisation upon exposure to zinc occurs rapidly. By 20 minutes post-pulse, the majority of cells exhibited numerous zincosomes as indicated by zinquin fluorescence. Therefore, *C. albicans* rapidly compartmentalises zinc within zincosomes in response to changes in environmental zinc.

We therefore turned our attention to ZnT-type transporter which, in contrast to Zip transporters (such as Zrt2), transport their substrate from the cytoplasm to either outside the cell, or into the lumen of intracellular compartments (Eide 2006).

Five *C. albicans* ZnT-type (PF01545) transporters were identified using FungiDB with sequence similarity to *S. cerevisiae* Mmt1/2 (orf19.52), Zrg17

(orf19.3769), Msc2 (orf19.3132), and Cot1/Zrc1 (orf19.1536), as well as a fifth protein encoded by orf19.3874 which does not have an orthologue in *S. cerevisiae* (**Table 1**). We therefore created deletion mutant for these five putative zinc transporter genes. For orf19.1536, we propose the common name, Zrc1.

Table 1. Identified ZnT-type transporter in *C. albicans* and their relationship with *S. cerevisiae*.

orf19.	Yeast best hit	E value	Yeast description
orf19.1536	Zrc1/Cot1	2.6e-94/5.3e-90	Vacuolar zinc importer
orf19.3874	None		
orf19.3769	Zrg17	1.5e-39	ER zinc import (heterodimer with MSc2)
orf19.3132	Msc2(/Zrc1 partial)	3e-76(/1.4e-28)	ER zinc import (heterodimer with Zrg17)
orf19.52	Mmt2/1	3.3e-62/3.9e-60	Mitochondrial iron import

To determine which ZnT-transporter may mediate zincosome compartmentalisation, wild type, *zrc1*Δ, orf19.3874Δ, orf19.3769Δ, orf19.3132Δ, and orf19.52Δ cells were pulsed with zinc for 20 minutes and stained with zinquin.

Figure 8A shows that the isogenic wild type exhibited a significant 5.6-fold increase in zinquin fluorescence following the zinc pulse. Deletion of orf19.3874, orf19.3769 or orf19.3132 had no effect in this assay. The orf19.52Δ mutant exhibited perturbed zincosome generation, but this was not significant under the conditions tested here. Deletion of *ZRC1*, on the other hand, strongly inhibited zincosome formation and this was restored to wild type levels by genetic complementation with a single copy of *ZRC1* (**Figure 8A**).

This screen indicated that the ZnT-type transporter, Zrc1, plays a role in zincosome formation. For wild type, *zrc1*Δ and *zrc1*Δ+*ZRC1* strains, the experiment was repeated and zincosome accumulation determined at 5, 10 and 20 minutes post-pulse by flow cytometry. **Figure 8B** shows that both wild type and *zrc1*Δ+*ZRC1* strains exhibited progressive increases in zinquin fluorescence following the zinc pulse, resulting in more than a 10-fold increase by 20 minutes compared to the pre-pulse condition. In contrast, *zrc1*Δ exhibited only a moderate (~3-fold) increase in fluorescence by 5 minutes, and the signal did not significantly increase at later time points. These data show that the ZnT-type transporter Zrc1 is required for zincosomal zinc accumulation in *C. albicans*. Interestingly, when we measured actual zinc uptake

within this shorter time period, cells took up less than 30% within 20 minutes, suggesting that these very early zincosome formation events (**Figure 7-8**) may be the result of intracellular zinc mobilisation, prior to significant cellular uptake (**Figure 2**). Indeed, we have very recently demonstrated that *C. albicans* undergoes very rapid (seconds) remobilisation of intracellular zinc pools upon changes in environmental zinc, in the absence of cellular uptake (Kjellerup, Winther et al. 2018).

The kinetics of zincosome formation in the model yeast *S. cerevisiae* have been reported to be similar to those described here, occurring within 5-20 minutes exposure of zinc-depleted cells to a zinc pulse (Devirgiliis, Murgia et al. 2004). However, the mechanistic basis of zincosomal zinc accumulation appears to be fundamentally different in these two species. *S. cerevisiae* encodes two orthologues of *C. albicans* Zrc1: Zrc1 and Cot1. However, single *zrc1*Δ, *cot1*Δ and *zrc1*Δ/*cot1*Δ double mutants exhibited wild type zincosome formation, suggesting that neither ScZrc1 nor its paralogue, Cot1, are involved in zincosome formation in *S. cerevisiae* (Devirgiliis, Murgia et al. 2004). In fact, *S. cerevisiae* Zrc1 instead plays a clear and important role in vacuolar zinc accumulation (Simm, Lahner et al. 2007).

We therefore sought to characterise the relationship between our novel Zrc1-zincosome pathway and vacuolar zinc in *C. albicans*. Co-staining cells with zinquin and the vacuolar membrane dye FM4-64 (Veses and Gow 2008) revealed that zincosomes are not found within the fungal vacuole in *C. albicans* but rather, close to the outer leaflet of the vacuolar membrane (**Figure 9A**). Given this relatively close spatial relationship, we next questioned whether Zrc1-dependent zincosomal zinc compartmentalisation was an upstream component of vacuolar zinc trafficking in *C. albicans*. We first established that *C. albicans* can sequester zinc within the vacuole using the fluorescent probe Zinpyr1 (**Figure 9B**). Interestingly, in our zinc-pulse experiment, *zrc1*Δ accumulated vacuolar zinc to the same levels as the wild type, even after extended incubation (**Figure 9C**). Therefore, under the conditions tested, Zrc1 in *C. albicans* is not essential for vacuolar zinc import.

Zrc1 has been reported to localise to the vacuole in *S. cerevisiae* and *C. neoformans* (Li and Kaplan 1998, Cho, Hu et al. 2018). However, our own analysis indicated that *C. albicans* Zrc1 is dispensable for vacuolar zinc import under the conditions tested here (**Figure 9C**). To test whether Zrc1 localises to the *C. albicans* vacuole, we tagged the protein at its C-terminus with a codon optimised Venus fluorescent protein. **Figure 10** shows that *C. albicans* Zrc1, unlike its *S. cerevisiae* and *C. neoformans* orthologues, does not localise predominantly to the vacuolar membrane, but instead to the internal membrane system, reminiscent of the endoplasmic reticulum. This localisation is more similar to that of

Schizosaccharomyces pombe Zhf1 which transports zinc into the endoplasmic reticulum (Clemens, Bloss et al. 2002).

Zrc1-dependent zincosomal detoxification is essential for adaptation to environmental zinc.

Given the importance of Zrc1-mediated vacuolar zinc detoxification in the model yeast *S. cerevisiae* and in the basidiomycete pathogen *C. neoformans*, we next questioned whether a relationship exists between Zrc1, zincosomes, and metal tolerance in *C. albicans*.

First, we screened *zrc1*Δ, as well as all other ZnT-transporter deficient mutants for sensitivity to $\log_{10}$-fold increases in Zn^{++}, Fe^{++}, Mn^{++} and Cu^{++} alone or in combination. We included the other mutant strains and other metals to test for potential redundancy and transporter promiscuity. We did not observe significant synergistic toxicity of the tested metals, however excess manganese protected cells from zinc toxicity. The mutant lacking orf19.3132 exhibited increased sensitivity to excess manganese and all strains exhibited relatively similar levels of iron and copper tolerance (**Supplementary Figure S7**).

Lack of Zrc1, on the other hand, resulted in approximately 100-fold increased Zn^{++}-sensitivity (**Figure 11A and S7**) and genetic complementation restored Zn^{++} tolerance back to wild type levels (**Figure 11A**). The observed Zn^{++} sensitivity of *C. albicans* observed in these experiments is likely due growth inhibition, rather than fungal killing. Indeed, we had to expose cells to molar concentrations of zinc to kill *C. albicans*. Although *zrc1*Δ was also hypersensitive to Zn^{++} killing (**Figure 11B**), it is unclear whether *C. albicans* will face such high levels of Zn^{++} in nature. On the other hand, sub-millimolar to millimolar concentrations are well within the physiological range *C. albicans* will likely face in its natural environment as a human commensal and pathogen. Therefore, *C. albicans* Zrc1 plays a crucial role in adaptation to environmental zinc.

To examine whether there was a link between Zrc1-dependent zinc tolerance and zincosome formation, we exposed cells to 1 mM Zn^{++} for 2 h and measured zinquin fluorescence. This was chosen because Zrc1 is essential for growth at this concentration (**Figure S7 and Figure 11A**) and, whilst it is tolerated by wild type cells, is close to toxicity. Wild type *C. albicans* cells exhibited a considerable (31-fold) increase in zinquin fluorescence in response to challenge with 1 mM Zn^{++}. This was significantly reduced in *zrc1*Δ and restored to wild type levels by genetic complementation with *ZRC1* (**Figure 12A**). Fluorescence microscopy revealed that

these quantitative measurements reflect zincosome formation in wild type and *zrc1*Δ+*ZRC1*, but not in *zrc1*Δ cells (**Figure 12B**). Therefore, Zrc1 plays a crucial role in zincosomal zinc compartmentalisation in response to both relatively minor fluctuations in zinc availability (**Figure 8**) and potentially toxic levels of heavy metal (**Figure 11A and 12**). Together these data suggest that Zrc1-dependent zincosome formation is important for *C. albicans* adaptation to environmental zinc.

The current study is amongst the first detailed reports of intracellular zinc trafficking in a human fungal pathogen. We therefore assessed whether *C. albicans* Zrc1 plays a role in virulence. For this we chose two different infection models. Insect larvae have been reported to accumulate high levels of zinc (Cai, Hu et al. 2017). We therefore first performed infection experiments on the commonly used *Galleria mellonella* larva. The majority of wild type and *zrc1*Δ+*ZRC1* infected larvae succumbed to infection within 2-3 days post infection. Strikingly, only a single *zrc1*Δ infected larvae died in these experiments, showing that Zrc1 is essential for virulence in this model (**Figure 13** & **S8**). Although *C. albicans* is not a known pathogen of insect larvae, this observation is interesting because it suggests that *Galleria* may possess a form of high-zinc nutritional immunity; a phenomenon which has been reported in mammals (Botella, Peyron et al. 2011) and, recently, in plants (Fones, McCurrach et al. 2016).

In mammals, inflammation and the acute phase response result in zinc trafficking to the liver in order to induce zincaemia (Liuzzi, Lichten et al. 2005). We therefore assessed the capacity of *zrc1*Δ to colonise the murine liver. As shown in **Figure 14**, *zrc1*Δ exhibited a clear and significant defect in liver colonisation compared to both wild type and *zrc1*Δ+*ZRC1*. In contrast, *zrc1*Δ exhibited the same kidney fungal burden as the wild type (**Figure S9**). *zrc1*Δ mice gained 5% body weight between day 1 and day 3 post infection, whilst wild type and *zrc1*Δ+*ZRC1* infected mice lost weight (1.2-3%). This, together with larval survival (**Figure 13**) and liver colonisation (**Figure 14**) data indicate that Zrc1 plays an important role in *C. albicans* virulence

In summary, we have described a novel pathway of zinc import and compartmentalisation in *C. albicans* and demonstrated the significance of these mechanisms for both microbial physiology and *in vivo* fitness. Interestingly, the cellular import pathway of this fungus appears to be highly similar to that of *A. fumigatus* and we have proposed an ecological-evolutionary framework which may explain some of the conservation and divergence that we observe in extant human fungal pathogenic species. We also demonstrate, that unlike any previously characterised pathogenic fungi, *C. albicans* assimilates zinc from environment to zincosomes using a Zrt1,2/Zrc1-dependent biphasic mechanism.

Methods

Strain construction

C. albicans strains used in this study are listed in **Table S1**. The triple-auxotrophic strain BWP17 complemented with plasmid CIp30 served as the isogenic wild type control in all experiments. Homozygous *C. albicans* mutants were constructed as described previously (Mayer, Wilson et al. 2012) and the primers used for this are listed in **Table S2**. Briefly, forward primers were designed with 104 bp homology to the immediate upstream region of the gene of interest, followed by a 22 bp sequence, with homology to the pFA plasmids, immediately upstream of the respective selective marker. Similarly, reverse primers were designed with 104 bp homology to the immediate downstream region of the gene of interest (reverse complement), followed by 24 bp sequence with homology to the pFA plasmids, downstream of the selective marker.

These long primers, together with plasmids pFA-HIS1 and pFA-ARG4 were used to create deletion constructs for each of the zinc transporter encoding genes and the two alleles of each gene sequentially deleted using the improved transformation protocol (Walther and Wendland 2003) and selecting for histidine or arginine prototrophy. In each case, correct integration was determined using gene-specific upstream and downstream primers, lying outside the site of homologous recombination to determine absence of wild type copy and presence of ::*HIS1* and ::*ARG4* alleles, as well as *HIS1* and *ARG4* specific internal primers to ensure correct integration of selective markers at both 5' and 3'. For double deletion of *ZRT1* and *ZRT2*, the *zrt1*Δ uridine auxotrophy was sequentially transformed by the SAT flipper technique to delete both copies of *ZRT2*. All these uridine auxotrophs were *URA3* complemented with *Nco*I-linearised CIp10 plasmid (Murad, Lee et al. 2000). For the double mutant, both *ZRT1* and *ZRT2* including up- and down- stream sequences were sub-cloned into CIp10. For *ZRT2* and *ZRC1*, the wild type alleles, together with the up- and down- stream intergenic regions were amplified from SC5314 gDNA with phusion polymerase and cloned into CIp10 at *Mlu*I and *Sal*I sites. Resulting plasmids were linearised with *Nco*I and used to complement the respective homozygous deletion mutants. For creation of the P_{ZRT1} and P_{ZRT2} GFP reporters, the upstream intergenic regions of *ZRT1* and *ZRT2* were amplified with phusion polymerase from SC5314 gDNA, cloned into CIp10-GFP (Cormack, Bertram et al. 1997) at *Xho*I and *Mlu*I sites and verified by sequencing. Resulting plasmids were linearised with *Nco*I and transformed into CAI4 for integration at the *RPS1* locus. In order to localise Zrc1, the protein was tagged at the C-terminus which is predicted to face the cytoplasm (Octopus (Viklund and Elofsson 2008), Phobius (Kall, Krogh et al. 2004), and TMHMM (Krogh, Larsson et al. 2001)), with a

Venus yellow fluorescent protein. The Venus sequence was codon optimised for expression in *C. albicans* and synthesised (GeneArt), flanked by Pfl23II (5') and BamHI (3'). The gene was subcloned into pFA-HIS1 at these sites generating pFA-HIS1-Venus. Both Venus and the *HIS1* cassette were amplified with primers ZRC1Ven-FG and ZRC1Ven-RG. These primers include 30 and 29 base pairs sequence homology to the template plasmid for amplification at the 3', and 100 and 99 bp homology to the *ZRC1* locus, to replace the *ZRC1* stop codon with Venus. The forward primer additionally contained ggtggtggt between locus- and plasmid- specific regions to introduce a 3 × glycine linker between Zrc1 and Venus. The amplified construct was used to replace the remaining stop codon in the *zrc1*Δ/*ZRC1* heterozygote which was then *URA3*-complemented with CIp10 as above. Resulting *zrc1*/*ZRC1*-VENUS strains were successfully cultured in the presence of 250 µM $ZnSO_4$ to ensure functionality of the tagged protein.

Strains and growth conditions

Strains were maintained on YPD agar [1% yeast extract, 2% myco-peptone, 2% D-glucose, 2% agar]. Liquid overnight cultures were grown in YPD or SD medium in a shaking incubator at 30°C and 200 rpm. Transformants were selected on SD agar supplemented with arginine, histidine and/or uridine (each 20 µg ml^{-1}) as required. For isolation of the *zrt2*Δ deletion mutant, selection plates were additionally supplemented with 1 mM $ZnSO_4$.

Escherichia coli was grown on LB agar [1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 2% agar] and overnight *E. coli* cultures were cultivated in a shaking incubator at 37°C and 200 rpm. For selection purposes 50 µg/ml ampicillin were added to solid or liquid LB medium.

Zinc limited media

To elicit severe zinc restriction, cells were precultured in YPD, washed three time in ultra-pure water and inoculated at OD_{600} (0.05) in 4 ml LZM (limited zinc medium with the components listed in **Table S3**) in plastic Universal flasks and incubated at 30°C, 200 rpm for three days. For growth experiments in 96 well plates, cells were inoculated to OD_{600} (0.001) and incubated for seven days. For pH-defined LZM, NaOH was added to alkalinise the medium as required and then the media was buffered with 50 mM Na-tartrate (pH4.5) MES (pH 5-6.5) or HEPES (pH 7-8). To determine *ZRT1* and *ZRT2* promoter activity, CAI4+CIp10, P_{ZRT1}-GFP and P_{ZRT2}-GFP strains were cultured overnight in YPD, washed three times with ultra-pure water and inoculated to OD_{600} =1 into pH-buffered LZM in black walled, clear-bottomed 96 well

plates and incubated for 16 h. Fluorescence was measured at 485/520 nm and background (CAI4+CIp10) fluorescence subtracted.

To determine metal toxicity, cells from an SD overnight culture were inoculated into SD medium containing indicated metals (starting OD_{600} 0.05) and OD_{600} determined following 24 h incubation at 30°C.

To determine fungal killing, cells were pre-grown in YPD for 24 h, washed twice in 1mM EDTA, twice in ddH20, then inoculated into fresh SD0 medium to a final OD600 = 0.5 for 24 h. After incubation, cells were adjusted to 10^5 cells/mL in SD0 + 1M ZnSO4 or, as a control, ddH20 for 3 h. Following incubation, cells were washed twice in ddH20, counted and then diluted to 1000 cells/mL in ddH20. Subsequently, 100µl of cell suspension (100 cells) was spread on YPD plates and incubated at 30˚C. Following incubation, CFUs were counted and compared to determine % survival.

Zinc uptake assays

Yeast. Cells were pre-grown in YPD for 24 h, washed twice in 1mM EDTA, twice in ddH20, then inoculated into fresh SD0 medium to a final OD600 = 0.5 for 24 h. After incubation, cells were adjusted to OD600 = 5 in SD0 medium and pulsed with 25µM ZnSO4 at 30˚C with shaking. At indicated time points, 50µl of the supernatant was collected and quantified for zinc using Abcam zinc assay kit.

Hyphae. Cells were pre-grown in YPD for 24 h, washed twice in 1mM EDTA, twice in ddH20, then inoculated into fresh SD25 medium to a final OD600 = 0.5 for 24 h. After incubation, cells were washed twice in 1mM EDTA, twice in ddH20, adjusted to 106 cells/mL in RPMI-0 (RPMI + 1mM EDTA, FeCl [6.17 µM], MnSO4 [13.24 µM] and CuSO4 [0.3 µM]), cultured in 12 well tissue culture plates and incubated at 37˚C and 5% CO2 for 24 h. Cells were then washed thrice in PBS and pulsed with RPMI (which was found to contain 3.61 µM Zn^{++}) + 25µM ZnSO4 (28.61µM Zn++ total) and incubated at 37˚C and 5% CO_2. At indicated time points, 50µl of the supernatant was collected and quantified for zinc using Abcam zinc assay kit.

Intracellular zinc visualisation

To assess zincosomal zinc compartmentalisation, cells were pregrown in YPD, 30°C, 200 rpm for one day, washed three times with distilled water and inoculated into minimal medium without added zinc “SD0” (2% glucose, 0.5% NH_4SO_4, 1X YNB without zinc [Formedium]). Whilst this medium does not contain added zinc, it also lacks a chelator, and thus represents moderate zinc depletion.

For microscopy and flow cytometry experiments, cell were inoculated to OD_{600} = 0.05. For the mutant screen, cells were inoculated to OD_{600} = 4. This was to ensure

that all strains were at a similar phase of growth, because the *zrt2*Δ mutant grows poorly in the absence of exogenous zinc.

These prestarved cells were then exposed to 25 μM $ZnSO_4$ for various times. Pre-pulsed and zinc-pulsed cells were fixed in Histofix, washed in PBS and stained with 25 μM zinquin ethyl ester (Sigma) for 40 minutes. Cells were again washed with PBS and analysed.

For microscopy, cells were additionally stained with Concanavalin A Alexafluor 647 to visualise the cell surface and analysed using DeltaVision microscope using appropriate filters (DAPI and RhTRITC). For the mutant screen, stained and unstained cells were added to the wells of a black-walled clear-bottomed 96 well plate and fluorescence measured at 355/475 nm using a FluoStar plate reader. Measurements were normalised by subtracting the background fluorescence of unstained cells from the stained samples. For flow cytometry, approximately 10^5 cells were measured using a BD LSRFortessa.

To localise zincosomes and the fungal vacuole, cells from an overnight YPD culture were washed with 1 mM EDTA and then ddH_2O, incubated in SD0 for 2-3 h. Cells were then incubated with 40 μM FM4-64 and 250 μM $ZnSO_4$ for 45 minutes, washed with EDTA then PBS, incubated zinquin for 45 minutes, washed and visualised using a DeltaVision fluorescent microscope.

To visualise vacuolar zinc, cells were pre-grown in YPD for 24 h, washed twice in PBS, and then stained with ZinPyr-1 (10 μM, 1 h, 37°C, 200 rpm, washed twice in PBS and incubated for a further 1 h). Following ZinPyr-1 staining, cells were stained with 40μM FM4-64 in YPD + 1mM $ZnSO_4$ for 40 min at 30˚C with shaking in the dark. Following this, cells were washed twice in YPD + 1mM ZnSO4 and subsequently inoculated into YPD + 1mM $ZnSO_4$ for 90 min without dye at 30˚C with shaking in the dark. Cells were then imaged using confocal microscopy.

To determine vacuolar import kinetics, wild type *zrc1*Δ cells were pre-grown in YPD for 24 h, washed twice in 1mM EDTA, twice in ddH20, and then inoculated into fresh SD0 medium to a final OD600 = 0.5 for 24 h. After incubation, cells were stained with 10μM ZinPyr-1 in PBS for 1 h at 37˚C with shaking in the dark. Cells were then washed twice in PBS and incubated for a further 1 h at 37˚C with shaking in the dark. Following incubation, cells were pulsed with 25μM $ZnSO_4$ in SD0 medium and incubated at 30˚C with shaking in the dark. At indicated time points, 100μl of sample was collected and transferred to a black-bottomed 96 well plate and quantified for ZinPyr-1 fluorescence using a fluorescent microplate reader.

Galleria infection model

Cells were pre-grown in YPD for 24 h, washed twice in 1mM EDTA, twice in ddH20, then inoculated into fresh SD25 medium to a final OD600 = 0.5 for 24 h. After incubation, cells were washed twice in 1mM EDTA, twice in PBS, adjusted to 5 x 10^6 cells/mL in PBS, and then 20 µl (1 x 10^5 cells/mL) injected into the abdominal pro-leg of larvae. Survival of the larvae was monitored on a 12 h basis post-infection.

Ethics statement

Mice were kept in the animal facility Umeå Centre for Compartive Biology (UCCB). All animal experiments in this study were carried out in strict accordance with the recommendations in the guide for the care and use of laboratory animals conformed to Swedish animal protection laws and applicable guidelines (djurskyddslagen 1988:534; djurskyddsförordningen 1988:539; djurskyddsmyndigheten DFS 2004:4) and with the Swedish animal protection law in a protocol approved by the local Ethical Committee (Umeå djurförsöksetiska nämnd) permit number A79-14.

Animal experiments

For analysis of in vivo fitness and virulence, C57BL/6 wild-type mice and S100A9-/- mice from the same background were infected intravenously with 5 x 10^5 CFUs per animal from logarithmically growing *C. albicans* cultures. Male and female mice were included in equal numbers for all infections, the average age of the mice was 12-16 weeks.

Mice were sacrificed by cervical dislocation after one or three days of infection. Kidneys and liver were harvested, homogenised and resulting cell suspensions were plated on YPD plates to determine fungal burden.

Neutrophils were isolated as described before (Ermert, Urban et al. 2009). Briefly, C57BL/6 mice were sacrificed by cervical dislocation and femurs and tibia of both hind limbs were dissected. Bone marrow was flushed out with RPMI1640 w/o PR supplemented with 100 µg/ml Carbenicillin and 50 µg/ml Kanamycin (Duchefa, both). After red blood cell lysis, neutrophils were purified using a discontinuous Percoll gradient of 52%, 69% and 78% PBS-buffered Percoll (GE Healtcare). Collected neutrophils from the 69%/78% interface were washed, resuspended in $HBSS^-$ and kept on ice. Prior to use, neutrophils were counted using a Vi-CELL cell counter (Beckman Coulter) and diluted to desired concentration in RPMI1640 w/o PR with antibiotics. All following assays were performed in this medium, if not stated otherwise.

Inhibitory capacity of mouse NETs was quantified as explained earlier (Bianchi, Niemiec et al. 2011). 5 x 10^5 mouse neutrophils were seeded into a 24-well plate. NET

formation was induced by 100 nM phorbol myristate acetate in the presence of 1 % (V/V) DNase-free mouse serum. Incubation occurred for 20-22 h at 37 °C with 5 % CO_2; NET induction was verified microscopically. NET supernatants were gently removed and 500 µl RPMI w/o PR were added containing 5 x 10^4 *Candida* cells to reach a multiplicity of infection (MOI) of 0.1. Incubation occurred for 20-22 h at 37 °C with 5 % CO_2. Fungal viability was assessed by metabolic activity (Hosseinzadeh and Urban 2013). Briefly, 0.33 mg/ml XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide; Invitrogen) and 27 µg/ml Co-enzyme Q_0 (Sigma-Aldrich) were added to each well. After an incubation of 15 min at 37 °C, the 450 nm absorbance of the supernatants was measured using a Fluostar Omega plate spectrometer (BMG Labtech).

Statistical analyses

Kidney fungal burden was analysed in IBM SPSS Statistics 24. Normality and homogeneity of variance were first tested, and ANOVA and Kruskal-Wallis tests performed as appropriate for each data set. For growth assays and expression analysis, data were analysed using GraphPad Prism and either Student's t-test or ANOVA performed as appropriate. For phylogenetic analyses, amino acid sequences were acquired from FungiDB (Stajich, Harris et al. 2012) or from the *Candida* Genome Database (Inglis, Arnaud et al. 2012). To construct phylogenetic trees Phylogeny.fr One Click was used (Dereeper, Audic et al. 2010, Niemiec, De Samber et al. 2015): Alignments were performed using MUSCLE, maximum likelihood calculated using PhyML and tree rendering using TreeDyn.

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We would like to thank all members of the Aberdeen Fungal Group and the Department of Microbial Pathogenicity Mechanisms for ever fruitful conversations. In particular Al Brown for insightful discussions and Donna MacCallum for assistance in statistical analysis of *in vivo* data. As well as the Microscopy and Histology and the Cytometry and *In vivo* Imaging Core Facilities at the University of Aberdeen.

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Figure Legends

Figure 1. pH-dependent functionality and regulation of Zrt1 and Zrt2 in *C. albicans*. (**A**) Zrt2 is essential in acidic medium. Indicated strains, precultered in YPD, were washed and cultured in SD (YNB+glucose) medium alone, or supplemented with 100 µM ZnSO4 or with 50 mM HEPES pH 7.4. Asterisks indicate statistical significance compared to the wild type; # indicates statistical significance compare to the *zrt2*Δ in SD; P <0.05. (**B**) *ZRT1* promoter activity is pH regulated and *ZRT2* is constitutively expressed under zinc limitation. (P_{ZRT1}-GFP and P_{ZRT2}-GFP reporter strains in LZM buffered to indicated pH values). LZM was used due to lower green autofluorescence. Experiment performed three times. (**C**) Double deletion of *ZRT1* and *ZRT2* precludes growth at both acidic and neutral alkaline pH. Strains were cultured as in (A) and growth kinetics measured over 48 h in a microtitre plate. Experiment performed twice in triplicate.

Figure 2. Zinc uptake by *C. albicans* is mediated by Zrt1 and Zrt2. (**A**) Indicated strains were cultured in low zinc medium (SD0, pH ~4.7), exposed to 25 µM $ZnSO_4$ and zinc acquisition determined at indicated time points by measuring how much zinc remained in the cell free supernatant. *C. albicans* wild type acquires all measurable zinc within 60 minute; *zrt2*Δ does not; complementation restored zinc acquisition to 68%. Experiment was performed three times. (**B**) Indicated strains were incubated in RPMI without zinc for 24 h, exposed to 25 µM $ZnSO_4$ and zinc acquisition determined as in panel A. Wild type cells acquire 74% of zinc by three hours; uptake is reduced by approximately 50% in *zrt1*Δ and *zrt2*Δ. *zrt1*Δ/*zrt2*Δ fails to take up zinc. Experiment performed twice. Data points have been shifted to the right to make them visible amongst strains.

Figure 3. P_{ZRT1} and P_{ZRT2} metallo-regulation is zinc specific. Excess (100 µM) zinc, but not iron, manganese or copper downregulate P_{ZRT1}-GFP and P_{ZRT2}-GFP. Experiment was performed three times. *(P <0.05) and *** (P <0.0001)= significantly different from LZM, Student's t-test.

Figure 4. Growth of *zrt2*Δ strains is specifically rescued by excess zinc. Indicated strains were cultured as in Figure 1 with zinc, iron, manganese (100 µM) or copper (20 µM) and growth kinetics measured over 36 h in a microtitre plate. Experiment performed twice in triplicate. Iron had a moderate inhibitory effect on *C. albicans* growth. Note that only zinc rescued growth of *zrt2*Δ strains.

Figure 5. *C. albicans* Zrt2 is required for kidney colonisation in the presence of functional calprotectin. Indicated mice strains were infected with indicated fungal strains and kidney colonisation determined by plating CFUs on day one and day three post-infection. At day three post-infection, *C. albicans* wild type kidney fungal burden had increased significantly by 6.5-fold (P = 0.034), Deletion of *ZRT2* precluded an increase in kidney fungal burden between day one and day three post-infection (P = 0.597), asterisk. Complementation of *zrt2*Δ with a single copy of *ZRT2* restored kidney colonisation at day three (4.5-fold higher than at day one, P = 0.004).

Figure 6. Zrt2 protects against calprotectin-dependent inhibition of fungal growth during *C. albicans*-neutrophil extracellular trap interaction. Indicated strains were incubated with wild type or S100A9-/- -derived NETs or in medium only. Following ~21 hours incubation, metabolic activity was determined by XTT assay. Activity in the presence of both NET groups was determined compared to control conditions in the absence of NETs. Experiment was performed three time. * indicates $P < 0.05$ and # not significantly different to wild type, Students t-test Data presented are fold reduction in activity due to the presence of calprotectin.

Figure 7. Kinetics of zincosome formation in *C. albicans*. Cells were incubated overnight in YNB-zinc-dropout medium (SD0) to deplete zincosomes and pulsed with 25 µM $ZnSO_4$ for indicated time points. Cells were then stained with zinquin to probe for zincosomal zinc and the cell wall stained with Concanavalin A conjugated to Alexa-647. Left hand column shows false colour overlay of cell wall (cyan) and zincosomes (magenta). Right hand column shows DIC; Experiment performed three times and representative images shown.

Figure 8. Zincosome formation is Zrt2 and Zrc1 dependent. (**A**) Zincosome screen. Wild type, ZnT deletion mutants, and *zrc1*Δ+*ZRC1* strains were pulsed with 25 µM zinc for 20 minutes and zincosome fluorescence determined by staining with zinquin. Prepulsed cells were also stained as control. Experiment was performed at least twice in duplicates and all data normalised to the post-pulse value of wild type. ANOVA was first performed on initial (pre-normalised data). Asterisks indicate statistical significance compared to wild type and to relevant deletion mutant *** $P < 0.0001$. (**B**) As panel A, except zinquin fluorescence kinetics was determined by flow cytometry. Experiment performed three times. *zrc1*Δ exhibits significantly reduced zinquin fluorescence compared to wild type and revertant at 20 minutes $P < 0.001$, ANOVA.

Figure 9. Relationship between zincosomes and vacuole in *C. albicans*. (**A**) Cells were co-stained with zinquin (zincosomes) and FM4-64, which stains the fungal vacuole membrane. Note that zincosomes are not intra-vacuolar. (**B**) The zinc-specific probe Zinpyr-1 can be used to detect vacuolar zinc in *C. albicans*. Cells were co-stained with Zinpyr-1 and FM4-64. Note that Zinpyr-1 stains vacuolar zinc in *C. albicans* (**C**) Zrc1 is not required for vacuolar zinc import. Cells were loaded with Zinpyr-1, pulsed with 25 μM zinc and Zinpyr-1 fluorescence determined at 0, 30, 60 and 180 minutes post pulse. Experiments performed at least twice.

Figure 10. Zrc1 exhibits intracellular membrane localisation. The remaining copy of Zrc1 in a *zrc1*Δ/*ZRC1* heterozygous mutant was tagged at its C-terminus with a codon optimised Venus yellow fluorescent protein. The resulting strain was incubated for 24 h in SD0, treated with 25 μM zinc for 20 minutes and imaged. Note that Zrc1 does not localise exclusively to the vacuole as is the case in *S. cerevisiae* and *C. neoformans*, but rather to the internal membrane system, reminiscent of the endoplasmic reticulum. Experiment was performed twice.

Figure 11. Zrc1 is essential for zinc detoxification. (**A**) Strains were cultured for 24 h in SD0 medium containing indicated zinc supplementation. Experiment performed at least three times in duplicate for zinc concentrations at 25 μM and above. *** indicates significant difference ($P < 0.0001$) compared to wild type and revertant, ANOVA. (**B**) Strains were precultured in SD0, challenged with 1 M $ZnSO_4$ for 3 h and viability assessed by measuring CFUs. ** indicates significant difference ($P < 0.01$) compared to wild type and revertant, ANOVA. Experiment performed three times for wild type and *zrc1*Δ and twice in duplicate for all three strains.

Figure 12. Relationship between Zrc1, zincosomes and zinc tolerance. (**A**) Cells were challenged with potentially toxic zinc (1 mM), stained with zinquin and fluorescence determined. $P < 0.0001$ compared to wild type and revertant. (**B**) Micrographs of cells treated as in A. Note that *zrc1*Δ is highly defective for zincosome formation in response to 1 mM $ZnSO_4$ – a condition under which wild type, but not *zrc1*Δ cells can growth (Figure S5).

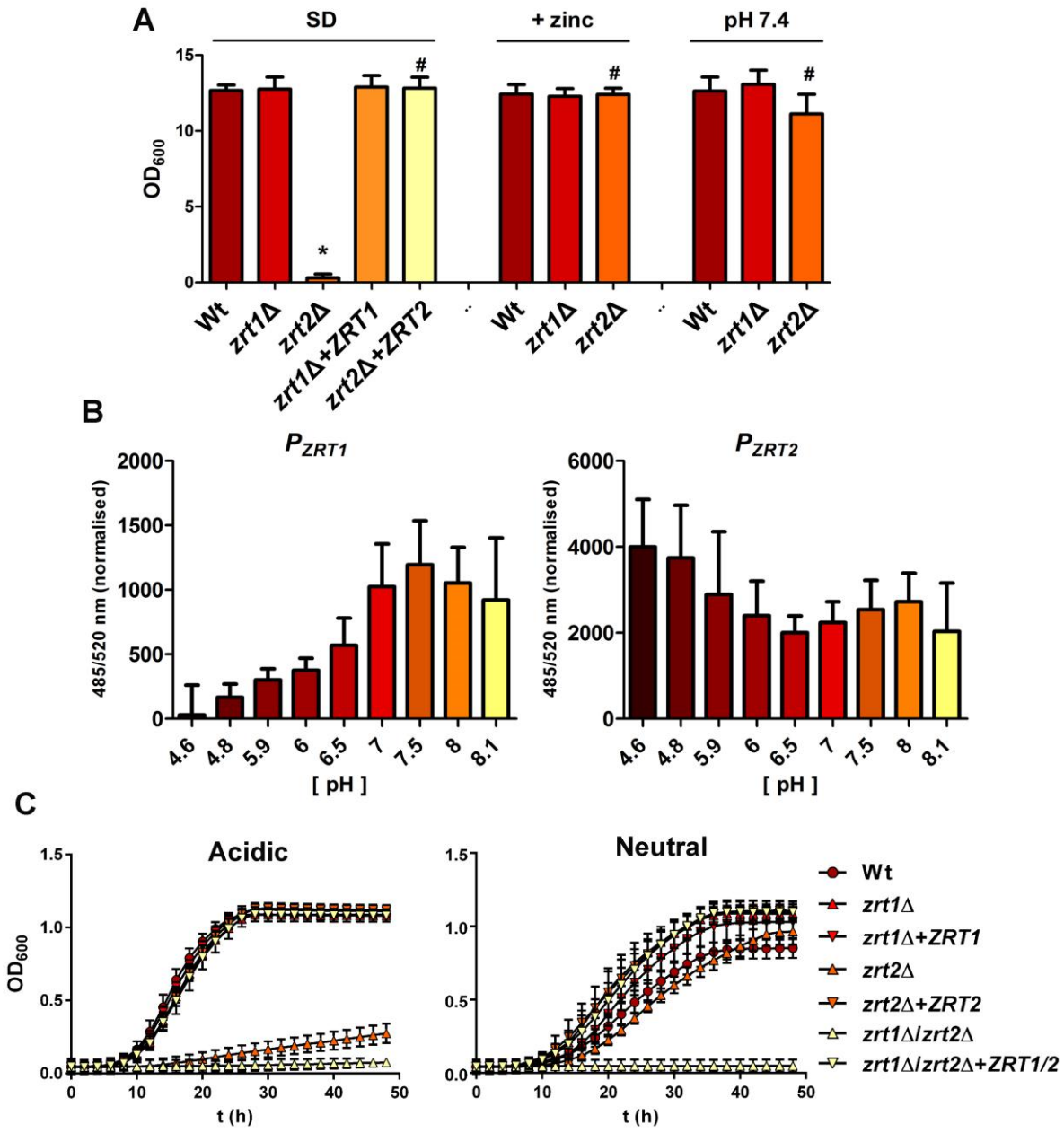
Figure 13. Zrc1 is required for virulence in a *Galleria* infection model. *Galleria* larvae (10 per group) were infected with 10^5 *C. albicans* cells and monitored every 12 h. Note that whilst wild type result in high mortality, only one *zrc1*Δ-infected larvae. Experiment performed twice - here, and in Figure S8. *zrc1*Δ is significantly attenuated

compared to wild type (P = 0.0001) and *zrc1*Δ+*ZRC1* (P = 0.0009), but not compared to PBS control (P = 0.3173); Log-rank (Mantel-Cox) test.

Figure 14. Zrc1 is essential for liver colonisation. Mice were infected with indicated fungal strains and liver colonisation determined by plating CFUs on day one and day three post-infection. Asterisks indicate significant difference compared to wild type and revertant, ANOVA.

Figures

Figure 1



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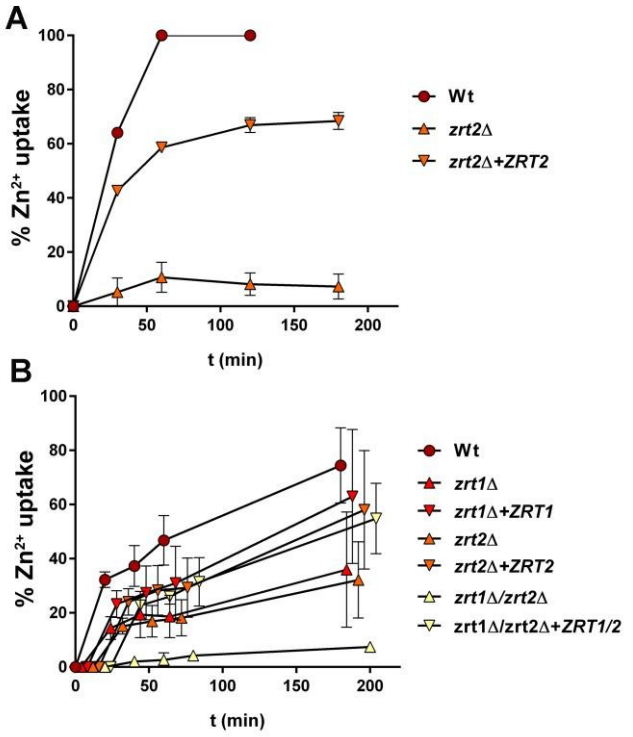
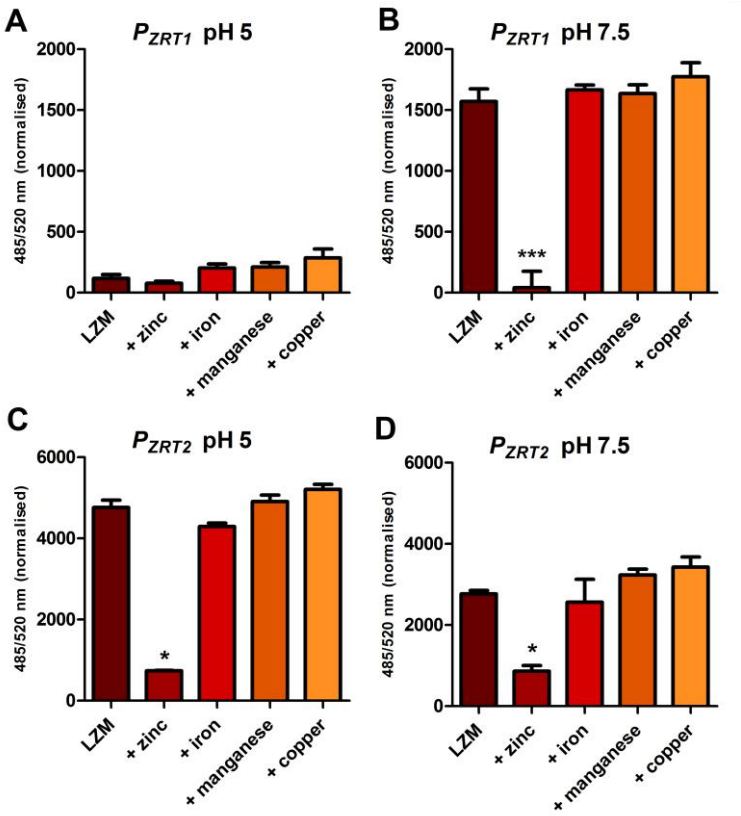


Figure 3



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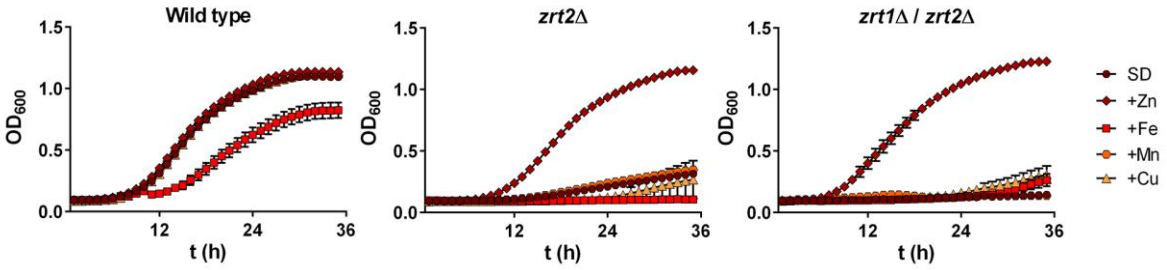

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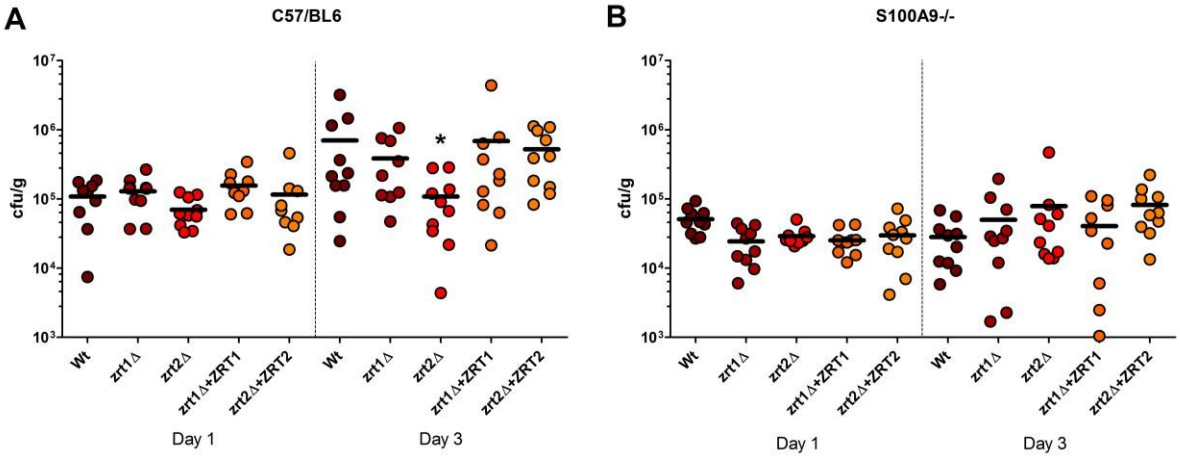

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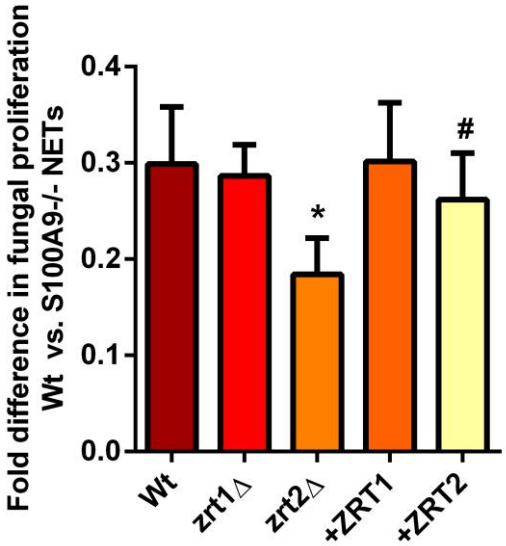

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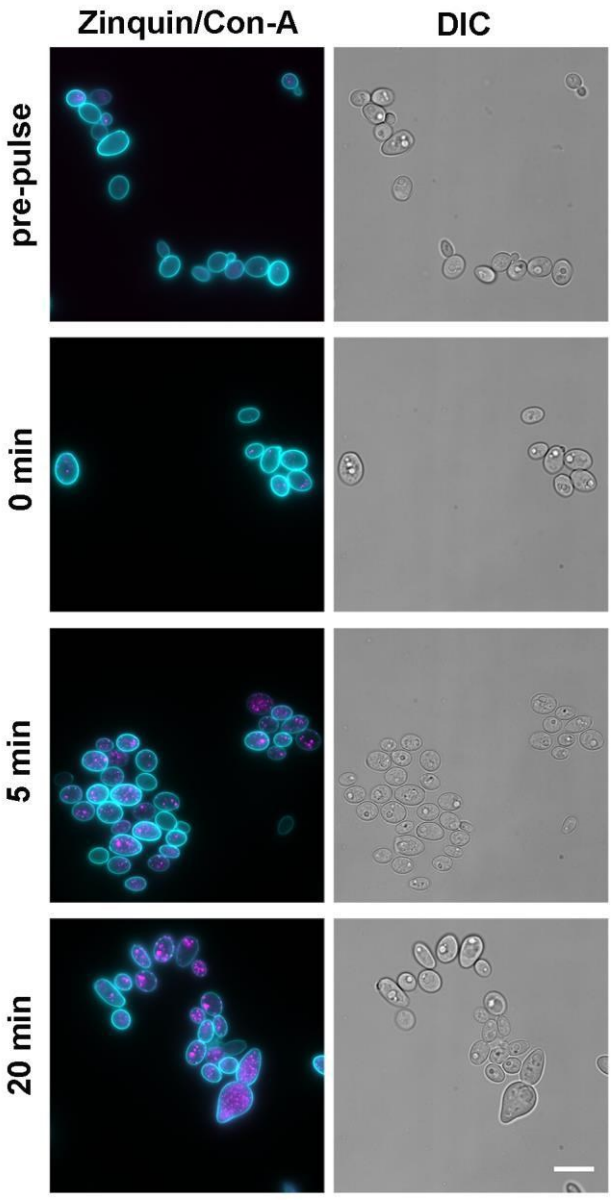

Figure 8

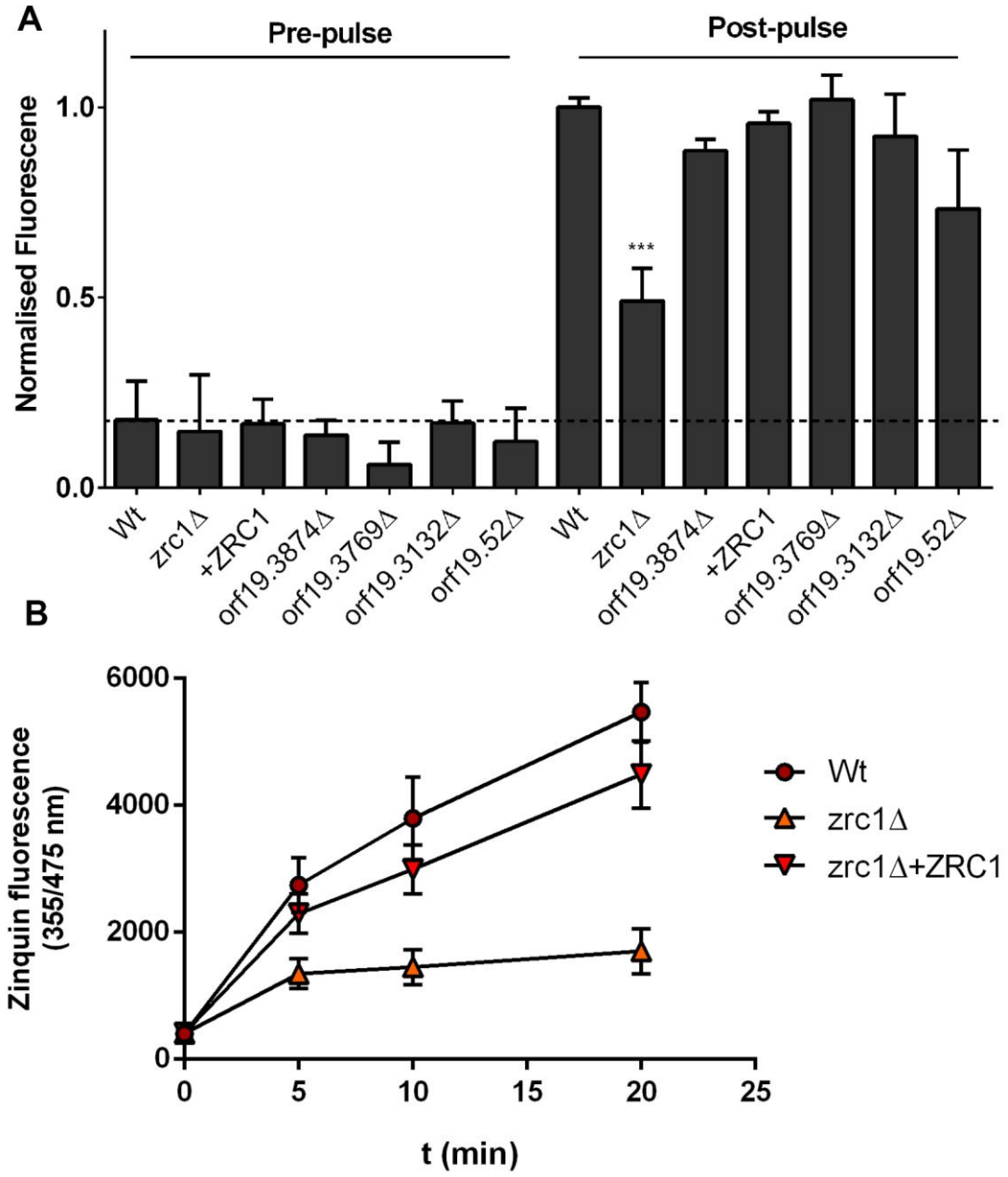
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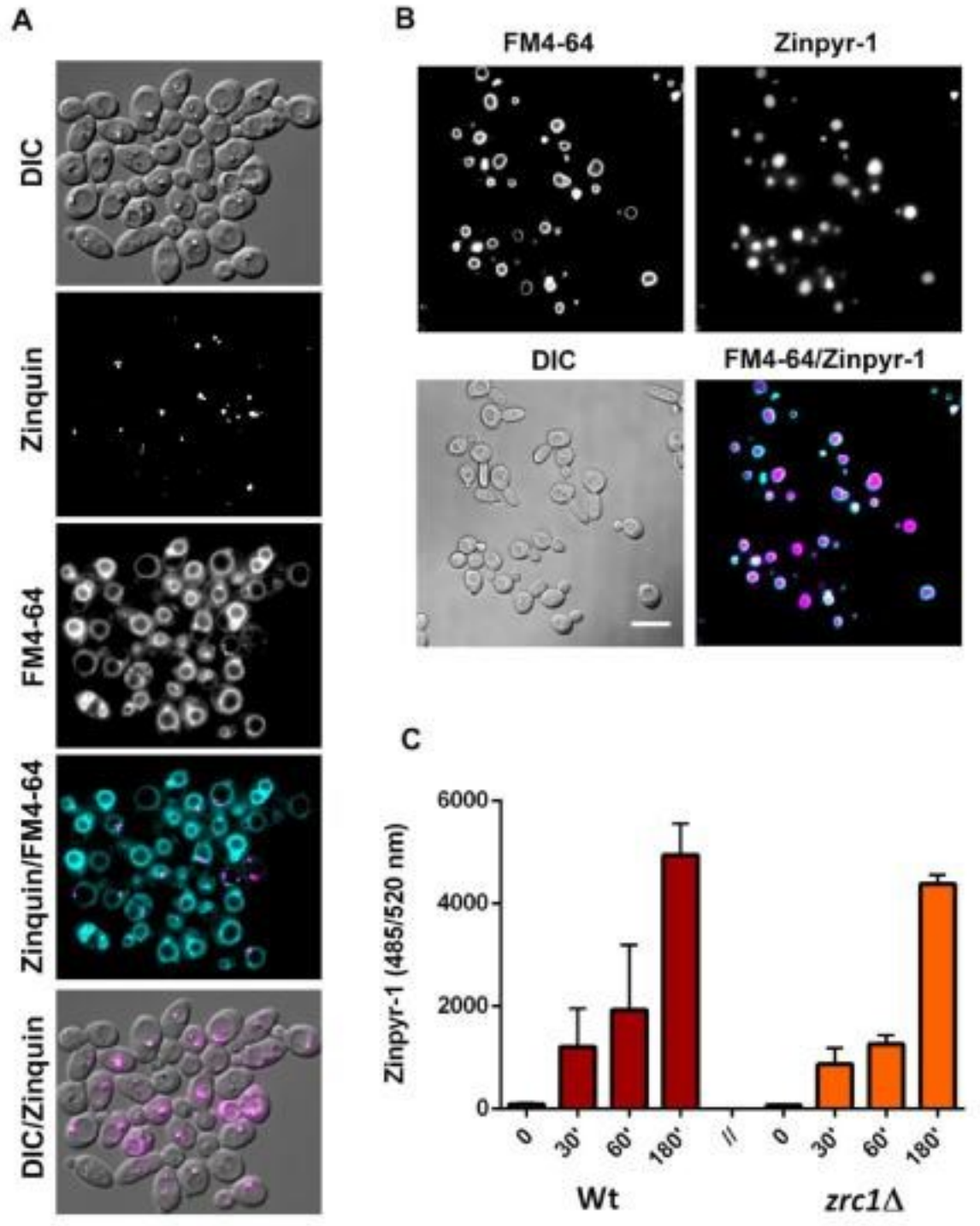
Figure 9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FM4-64/Zinpyr-1
C
Zinpyr-1 (485/520 nm)
6000
4000
2000
0
0
30'
60'
180'
//
0
30'
60'
180'
Wt
zrc1Δ

Figure 10

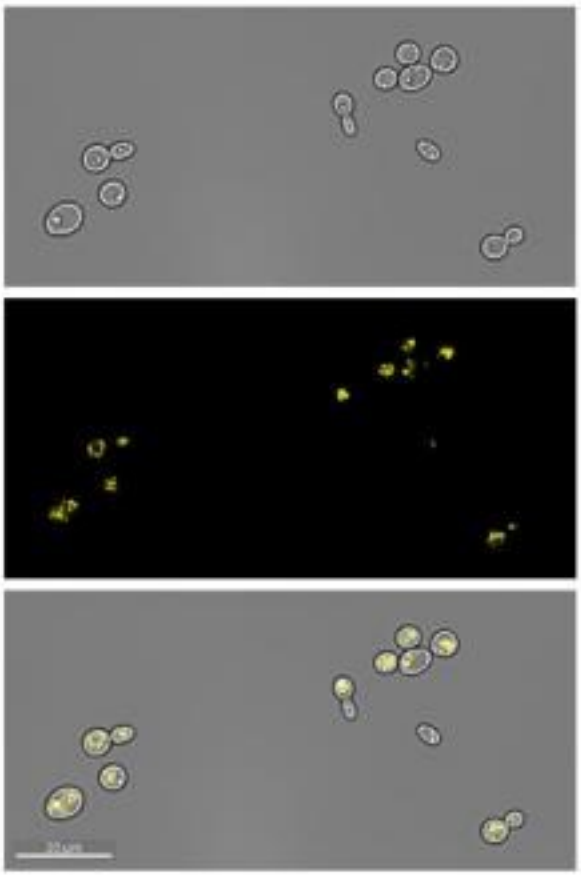


Figure 11

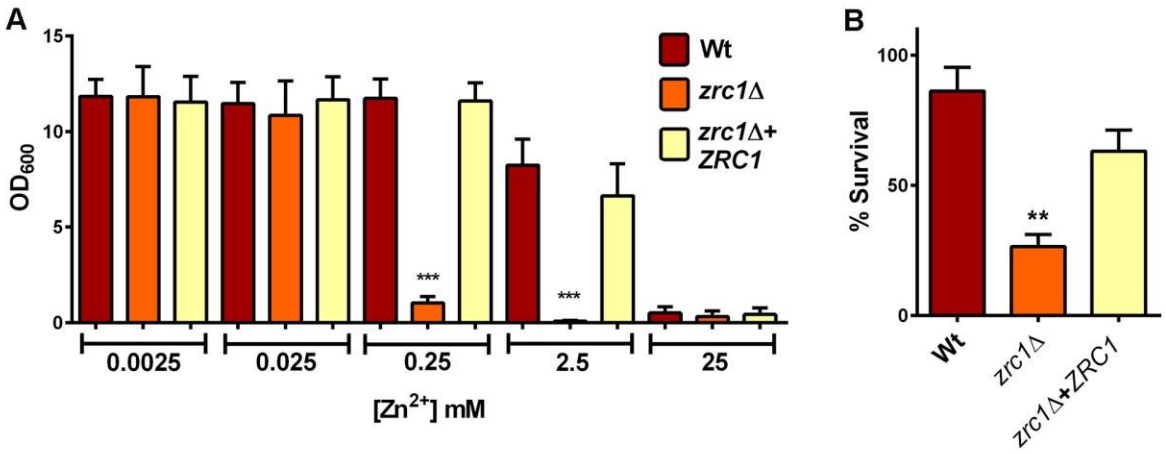


Figure 12

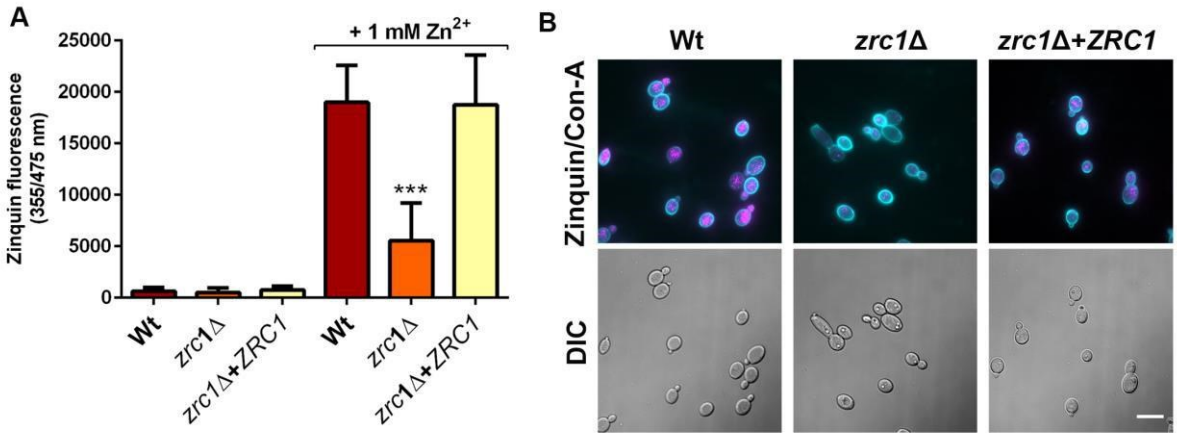


Figure 13

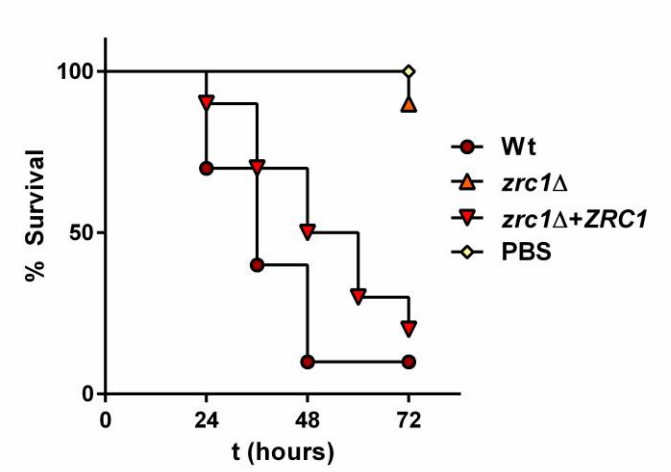
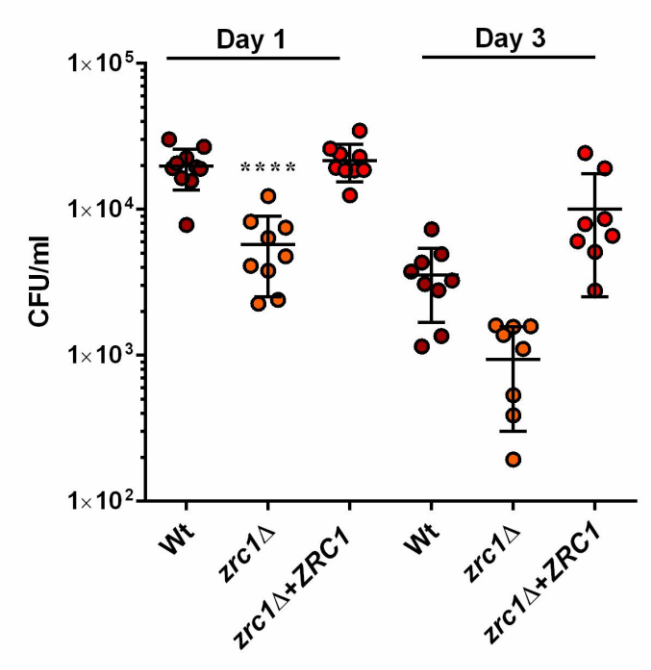


Figure 14


Day 1
Day 3
1×10^5
1×10^4
1×10^3
1×10^2
CFU/ml
****
Wt
zrc1Δ
zrc1Δ+ZRC1
Wt
zrc1Δ
zrc1Δ+ZRC1

Supporting Information Legends and Tables

Figure S1. Morphogenesis analysis. Indicated strains were inoculated into cell culture plates containing liquid 10% fetal calf serum (**a**), RPMI (**b**), or Spider (**c**) media, incubated at 37°C and imaged at indicated times. Alternatively, individual cells were spread onto 2% agar plates containing 10% fetal calf serum (**d**) or 10% RPMI medium (**e**), incubated at 37°C and resultant colonies imaged at day 6. All experiment performed at least twice.

Figure S2. Biofilm formation. Biofilms formed in RPMI (**a**, **b**), SD (**c**) or Spider (**d**) media and metabolic activity measured at 1.5 and 24 h (**a**, **c**) or biomass determined at 72 h. Experiment performed twice in triplicate.

Figure S3. Zrt2-dependence is bypassed at pH 7 and above. Strains from a YPD overnight culture were washed, inoculated into LZM at an OD_{600} of 0.005 and incubated at 30°C for seven days. (**A**) Growth recovery of *zrt2*Δ occurs at pH 7.0 and above. (**B**) Growth of all strains in LZM is recovered by addition of zinc (500 μM). Experiments were performed three times. * indicates statistical difference compared to wild type; # indicates statistical difference compared to mutant ($P < 0.05$, Student's t-test).

Figure S4. Relationship between environmental pH and zinc import copy number. (**a**) Map of soil acidity in the contiguous USA from the BONAP website (http://www.bonap.org/), reproduced with permission from Greg Schmidt, 2008, and includes data from the USDA Natural Resource Conservation Service. Pink colouring shows areas with high percentages (50-100%) of acidic soil (pH <6). Endemicity data for *C. immitis* (blue) and *H. capsulatum* from are superimposed. Panel (**a**) is inspired from our previous analysis in (Wilson 2015). (**b**) Phylogenetic tree of predicted plasma membrane zinc transporters in *C. albicans*, *C. parapsilosis*, *H. capsulatum*, *C. neoformans* and *M. globosa*, note expansion of Zrt2 orthologues in *C. parapsilosis*.

Figure S5. Phylogenetic relationship of zinc transporters in human fungal pathogens. All Zip-type proteins (PF02535) from *S. cerevisiae*, *C. albicans*, *A. fumigatus*, *C. neoformans,* and *C. gattii*. Red circle denotes demonstrated role in pathogenicity in relevant invasive fungal infection model; blue asterisks denote no/minor role in virulence; yellow diamonds denote redundancy.

Figure S6. Zrt2 protects against calprotectin-dependent inhibition of fungal growth during *C. albicans*-neutrophil extracellular trap interaction. Indicated

strains were incubated with wild type or S100A9-/- -derived NETs or in medium only. Following ~21 hours incubation, metabolic activity was determined by XTT assay. Activity in the presence of both NET groups was determined compared to control conditions in the absence of NETs. Experiment was performed three time. Shown are the actual measurements used to generate the relative activity presented in **Figure 5**.

S7. Effect of zinc and manganese, copper or iron on the growth of wild-type *C. albicans* and ZnT deletion mutants. Optical densities of SD overnight cultures were adjusted to 0.05 than incubated for 24 hrs in SD media containing indicated metal concentrations. Data are the mean of two independent experiments, performed in duplicate. Standard deviation (SD) values are shown in the right hand column.

Figure S8. Zrc1 is required for virulence in a *Galleria* infection model. *Galleria* larvae (10 per group) were infected with 10^5 *C. albicans* cells and monitored every 12 h. Note that whilst wild type result in high mortality, *zrc1*Δ-infected larvae were not killed. Experiment performed twice - here, and in Figure 13.

Figure S9. Zrc1 is dispensable for mouse kidney colonisation. Kidney fungal burden from the mouse infection experiment reported in Figure 12. No significant differences between strains.

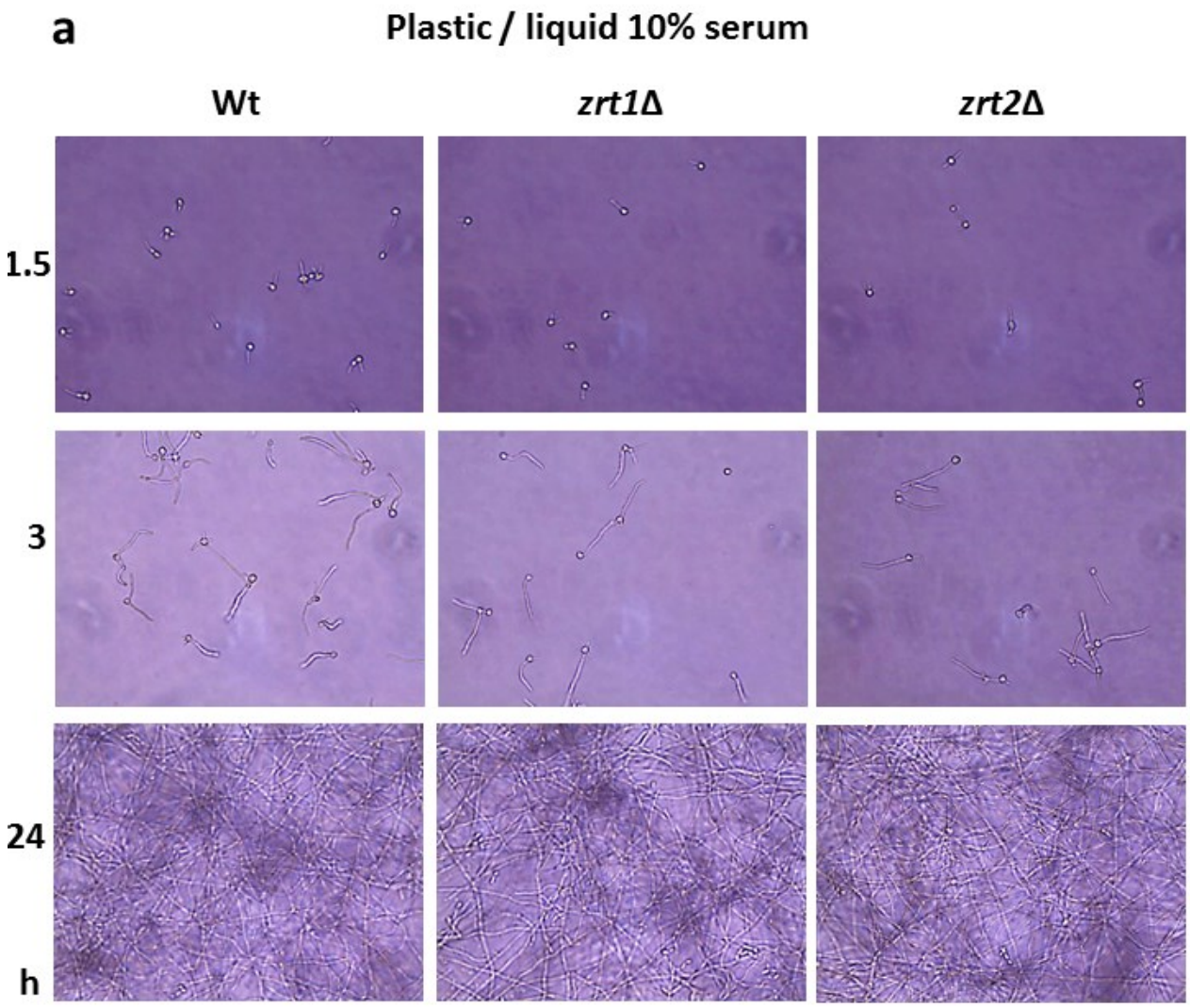
Table S1. Strains used in this study. All homozygous mutant strains created in the BWP17 (ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG) background. GFP reporters created in the CAI4 strain background.

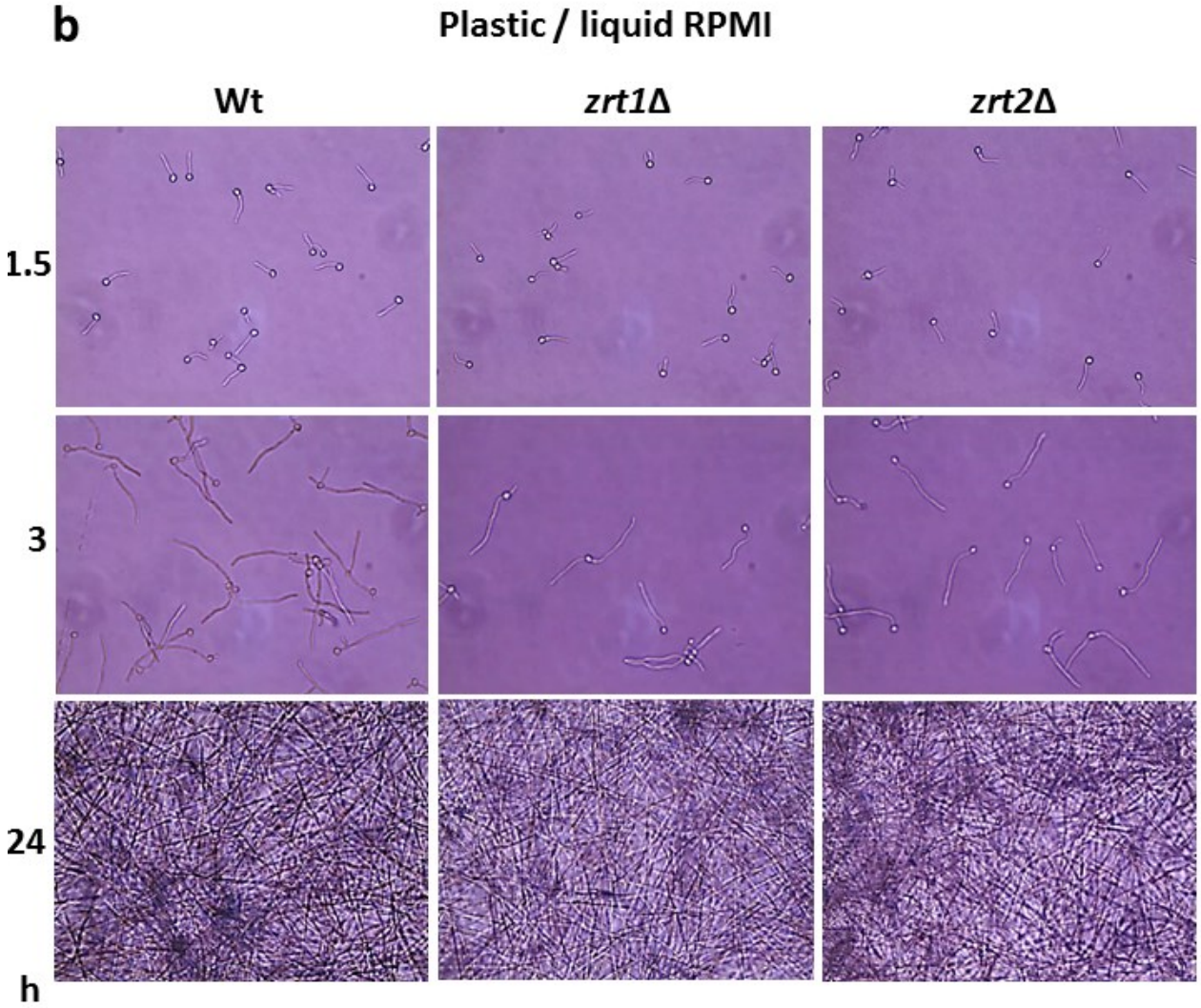
Table S2. Primers used in this study. FG and RG were used for deletion construct generation, pFA plasmid annealing site in lowercase; F1, R1 and Int for genotyping; RecF and RecR for revertant construction, restriction sites underlined.

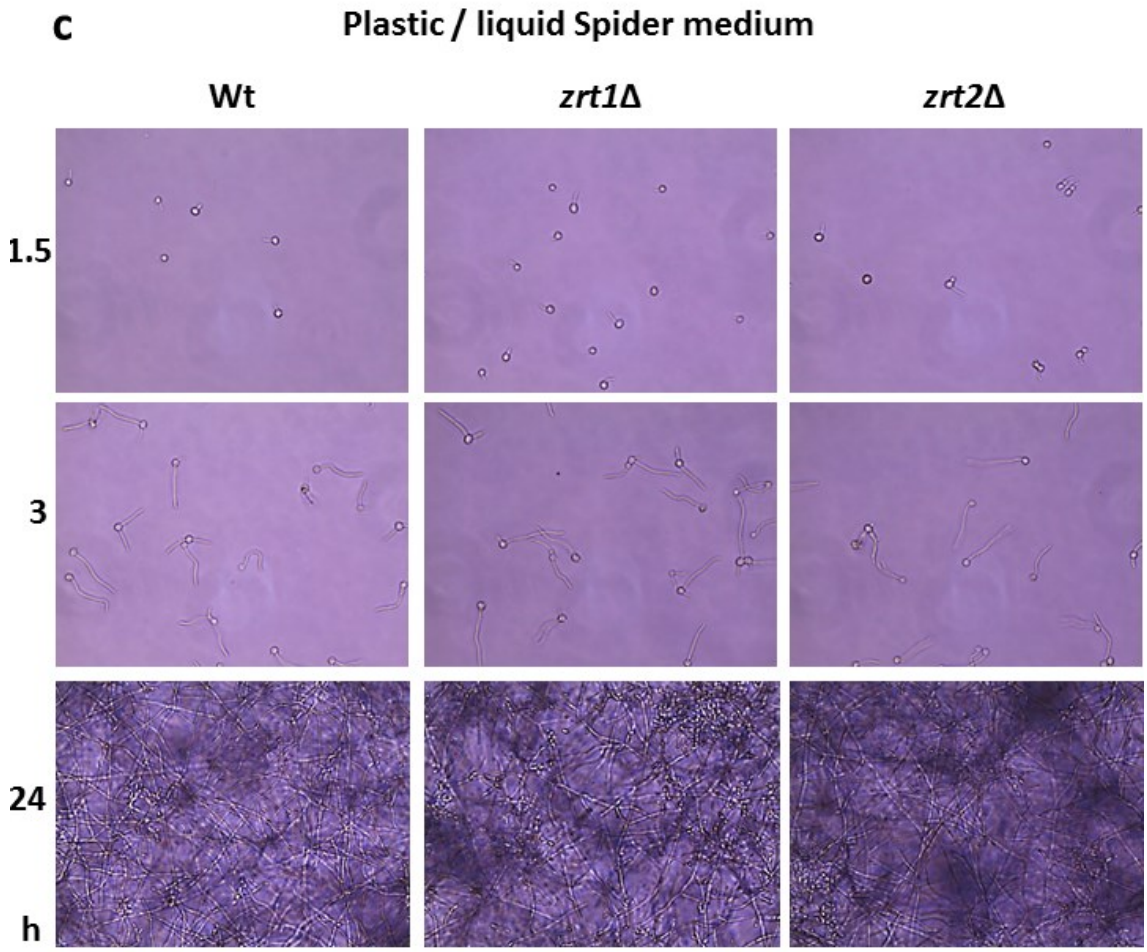
Table S3. LZM medium composition. EDTA (1) and sodium citrate (7) stocks were first adjusted to pH 8 and pH 4.2 respectively. Prepared medium was supplemented with FeCl (6.17 μM), MnSO4 (13.24 μM) and CuSO4 (0.3 μM).

Supplementary Figures and Tables

Figure S1




b
Plastic / liquid RPMI
Wt
zrt1Δ
zrt2Δ
1.5
3
24
h


c
Plastic / liquid Spider medium
Wt
zrt1Δ
zrt2Δ
1.5
3
24
h

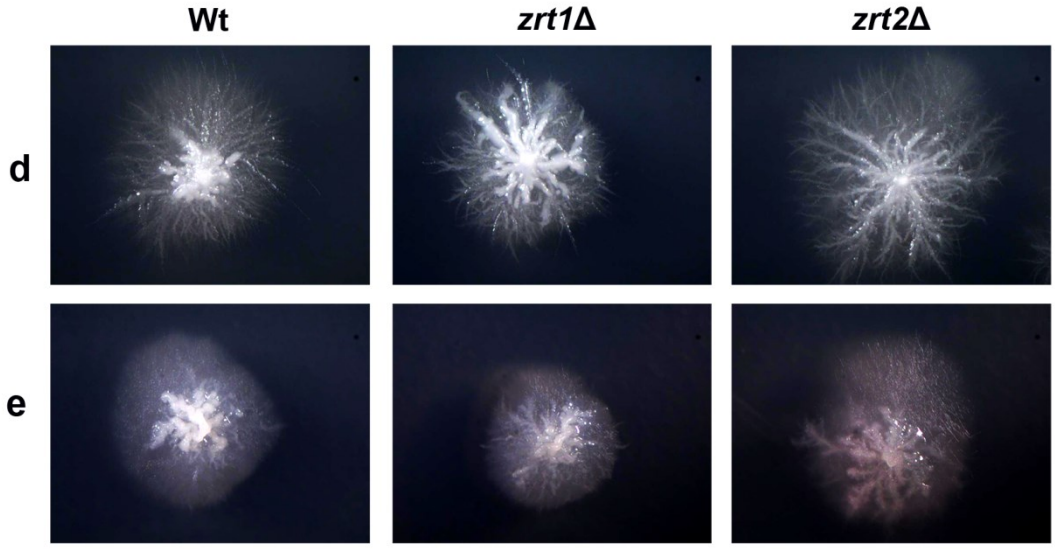

Wt
zrt1Δ
zrt2Δ
d
e

Figure S2

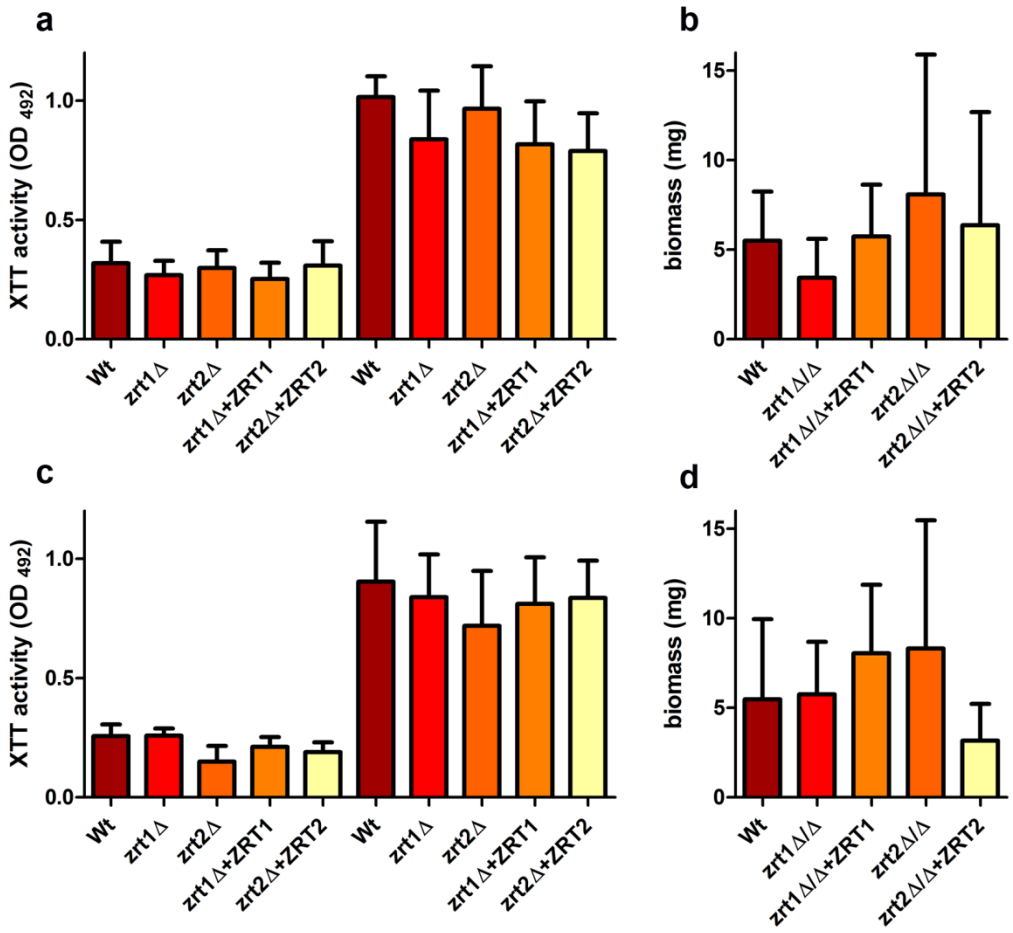

a
b
c
d
XTT activity (OD 492)
biomass (mg)
0.0
0.5
1.0
0
5
10
15
Wt
zrt1Δ
zrt2Δ
zrt1Δ+ZRT1
zrt2Δ+ZRT2
zrt1Δ/Δ
zrt1Δ/Δ+ZRT1
zrt2Δ/Δ
zrt2Δ/Δ+ZRT2

Figure S3

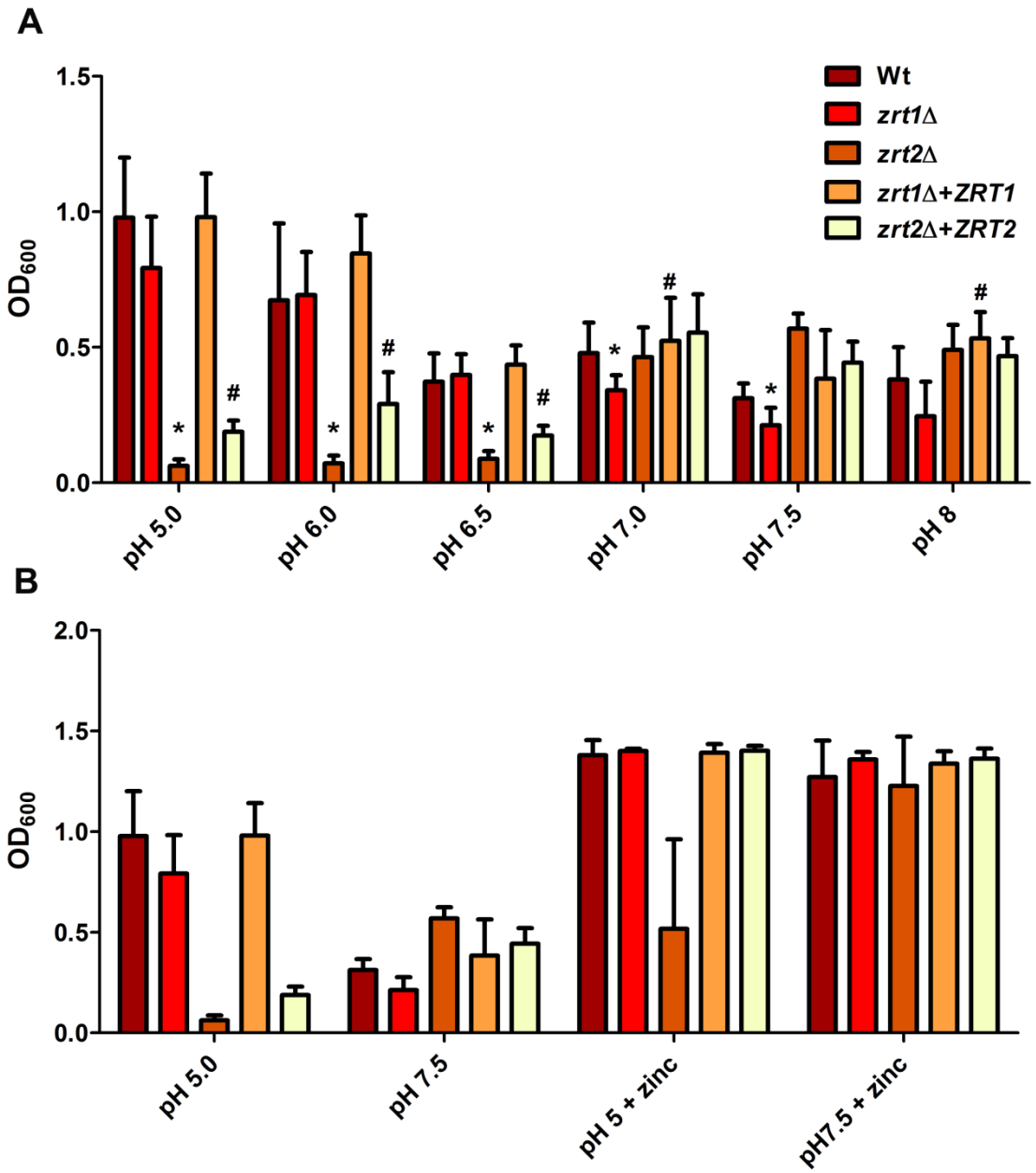

A
Wt
zrt1Δ
zrt2Δ
zrt1Δ+ZRT1
zrt2Δ+ZRT2
OD600
1.5
1.0
0.5
0.0
*
#
pH 5.0
pH 6.0
pH 6.5
pH 7.0
pH 7.5
pH 8
B
OD600
2.0
1.5
1.0
0.5
0.0
pH 5.0
pH 7.5
pH 5 + zinc
pH7.5 + zinc

Figure S4

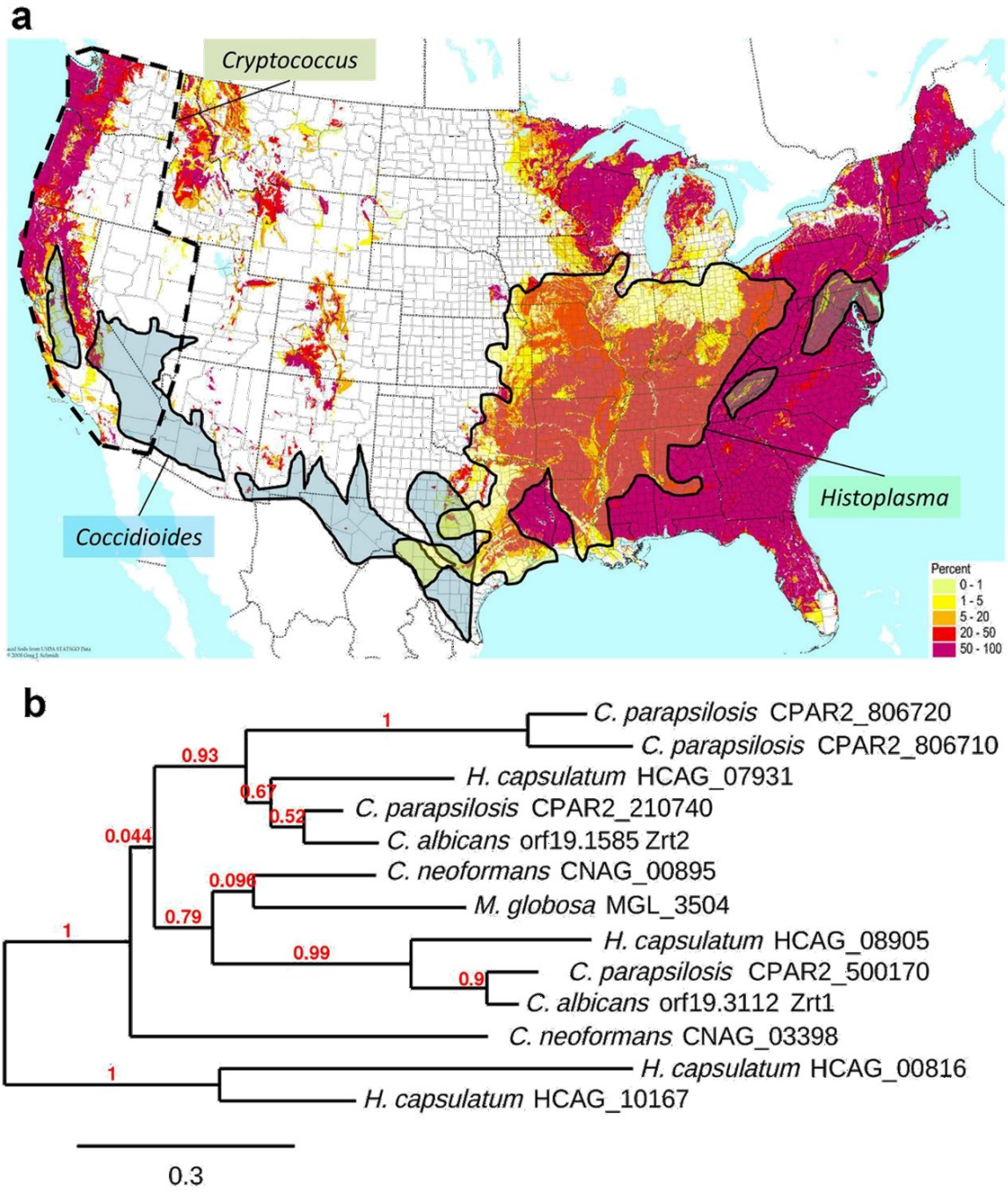

a
Cryptococcus
Coccidioides
Histoplasma
Percent
0 - 1
1 - 5
5 - 20
20 - 50
50 - 100
b
C. parapsilosis CPAR2_806720
C. parapsilosis CPAR2_806710
H. capsulatum HCAG_07931
C. parapsilosis CPAR2_210740
C. albicans orf19.1585 Zrt2
C. neoformans CNAG_00895
M. globosa MGL_3504
H. capsulatum HCAG_08905
C. parapsilosis CPAR2_500170
C. albicans orf19.3112 Zrt1
C. neoformans CNAG_03398
H. capsulatum HCAG_00816
H. capsulatum HCAG_10167
1
0.93
0.67
0.52
0.044
0.096
0.79
1
0.99
0.9
1
0.3

Figure S5

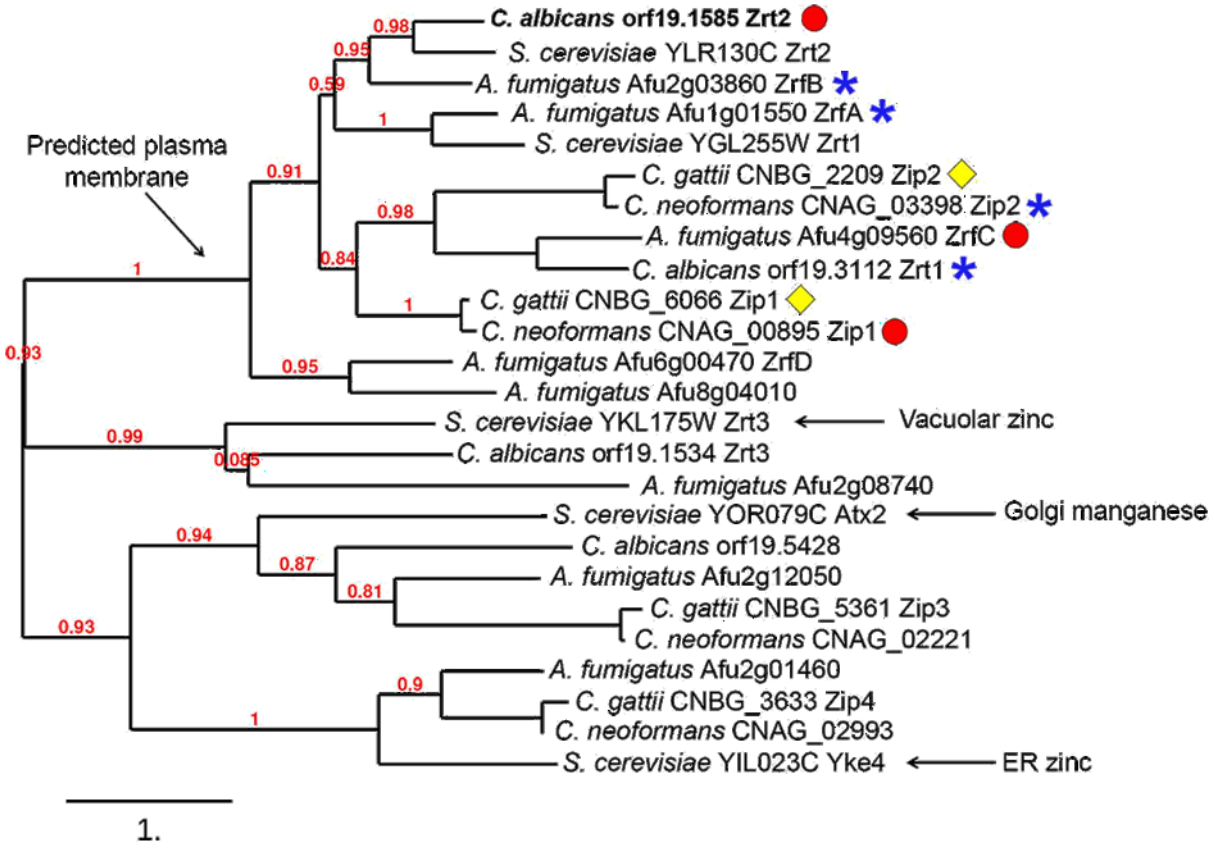


Figure S6

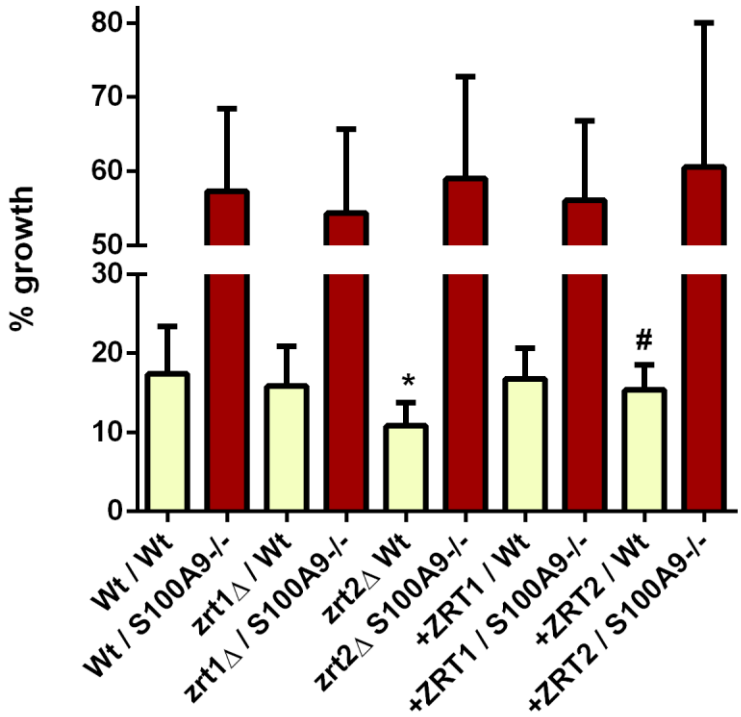
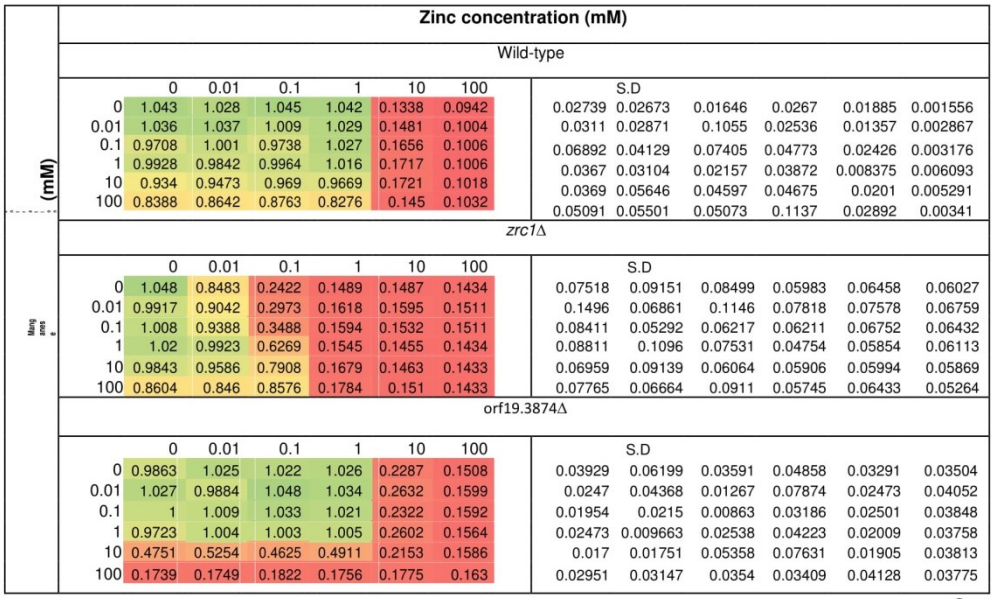


Figure S7



Zinc concentration (mM)

Wild-type

(mM)	0	0.01	0.1	1	10	100	S.D					
0	1.043	1.028	1.045	1.042	0.1338	0.0942	0.02739	0.02673	0.01646	0.0267	0.01885	0.001556
0.01	1.036	1.037	1.009	1.029	0.1481	0.1004	0.0311	0.02871	0.1055	0.02536	0.01357	0.002867
0.1	0.9708	1.001	0.9738	1.027	0.1656	0.1006	0.06892	0.04129	0.07405	0.04773	0.02426	0.003176
1	0.9928	0.9842	0.9964	1.016	0.1717	0.1006	0.0367	0.03104	0.02157	0.03872	0.008375	0.006093
10	0.934	0.9473	0.969	0.9669	0.1721	0.1018	0.0369	0.05646	0.04597	0.04675	0.0201	0.005291
100	0.8388	0.8642	0.8763	0.8276	0.145	0.1032	0.05091	0.05501	0.05073	0.1137	0.02892	0.00341

zrc1Δ

Manganese	0	0.01	0.1	1	10	100	S.D					
0	1.048	0.8483	0.2422	0.1489	0.1487	0.1434	0.07518	0.09151	0.08499	0.05983	0.06458	0.06027
0.01	0.9917	0.9042	0.2973	0.1618	0.1595	0.1511	0.1496	0.06861	0.1146	0.07818	0.07578	0.06759
0.1	1.008	0.9388	0.3488	0.1594	0.1532	0.1511	0.08411	0.05292	0.06217	0.06211	0.06752	0.06432
1	1.02	0.9923	0.6269	0.1545	0.1455	0.1434	0.08811	0.1096	0.07531	0.04754	0.05854	0.06113
10	0.9843	0.9586	0.7908	0.1679	0.1463	0.1433	0.06959	0.09139	0.06064	0.05906	0.05994	0.05869
100	0.8604	0.846	0.8576	0.1784	0.151	0.1433	0.07765	0.06664	0.0911	0.05745	0.06433	0.05264

orf19.3874Δ

	0	0.01	0.1	1	10	100	S.D					
0	0.9863	1.025	1.022	1.026	0.2287	0.1508	0.03929	0.06199	0.03591	0.04858	0.03291	0.03504
0.01	1.027	0.9884	1.048	1.034	0.2632	0.1599	0.0247	0.04368	0.01267	0.07874	0.02473	0.04052
0.1	1	1.009	1.033	1.021	0.2322	0.1592	0.01954	0.0215	0.00863	0.03186	0.02501	0.03848
1	0.9723	1.004	1.003	1.005	0.2602	0.1564	0.02473	0.009663	0.02538	0.04223	0.02009	0.03758
10	0.4751	0.5254	0.4625	0.4911	0.2153	0.1586	0.017	0.01751	0.05358	0.07631	0.01905	0.03813
100	0.1739	0.1749	0.1822	0.1756	0.1775	0.163	0.02951	0.03147	0.0354	0.03409	0.04128	0.03775

Cont.

Zinc concentration (mM)

orf19.3769Δ

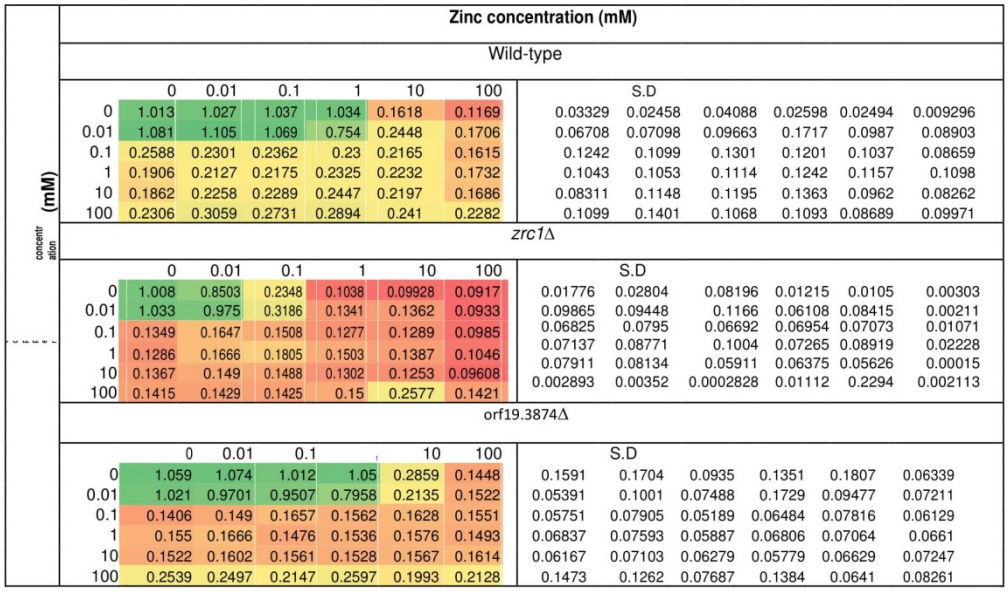
Manganese concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9722	1.014	1.015	0.9893	0.1756	0.1339	0.02768	0.01072	0.01468	0.02707	0.02406	0.07916
0.01	1.023	1.09	1.076	1.05	0.2668	0.138	0.1018	0.1115	0.06696	0.1138	0.1092	0.08174
0.1	1.02	1.129	1.135	1.1	0.3163	0.1513	0.09357	0.1025	0.04543	0.05549	0.1216	0.1051
1	1.033	1.113	1.107	1.158	0.3365	0.1423	0.1086	0.05611	0.06935	0.02049	0.1055	0.09253
10	1.006	1.059	1.092	1.099	0.3468	0.1074	0.1058	0.07745	0.02773	0.03425	0.1191	0.01904
100	0.8707	0.9228	0.9691	0.9641	0.254	0.1016	0.08373	0.07776	0.05621	0.0649	0.07957	0.001791

orf19.3132Δ

	0	0.01	0.1	1	10	100	S.D					
0	0.9525	0.9739	0.9631	0.9555	0.2052	0.1023	0.03748	0.0189	0.04237	0.04416	0.07187	0.01182
0.01	0.9841	0.9754	0.974	0.9756	0.1958	0.1066	0.03878	0.02773	0.02682	0.006616	0.009643	0.01527
0.1	0.953	0.9624	0.954	0.991	0.1956	0.1104	0.05977	0.01649	0.06982	0.0264	0.01171	0.01334
1	0.9334	0.9713	0.9781	0.9929	0.2078	0.1059	0.04915	0.02764	0.03003	0.0398	0.0108	0.01208
10	0.9193	0.9168	0.9294	0.9621	0.1852	0.1171	0.02042	0.02066	0.02691	0.02298	0.01998	0.01485
100	0.8044	0.8358	0.8048	0.8403	0.1669	0.1111	0.05876	0.003424	0.06754	0.0382	0.007115	0.01255

orf19.52Δ

	0	0.01	0.1	1	10	100	S.D					
0	1.105	1.07	1.112	1.069	0.3326	0.2417	0.0467	0.06284	0.05159	0.07308	0.05982	0.05575
0.01	1.104	1.109	1.096	1.124	0.3614	0.2289	0.03796	0.028	0.05515	0.05862	0.04834	0.01599
0.1	1.112	1.081	1.105	1.092	0.3776	0.2199	0.0921	0.0527	0.02678	0.06579	0.06682	0.02322
1	1.062	1.068	1.077	1.076	0.4115	0.2205	0.05456	0.03452	0.02559	0.05161	0.04312	0.02161
10	1.019	1.018	1.021	1.002	0.4008	0.2449	0.0533	0.04314	0.05197	0.1593	0.06464	0.04728
100	0.9198	0.9002	0.8503	0.9246	0.3791	0.213	0.06446	0.04215	0.1855	0.07723	0.07019	0.01864



Zinc concentration (mM)

Wild-type

(mM)	0	0.01	0.1	1	10	100	S.D					
0	1.013	1.027	1.037	1.034	0.1618	0.1169	0.03329	0.02458	0.04088	0.02598	0.02494	0.009296
0.01	1.081	1.105	1.069	0.754	0.2448	0.1706	0.06708	0.07098	0.09663	0.1717	0.0987	0.08903
0.1	0.2588	0.2301	0.2362	0.23	0.2165	0.1615	0.1242	0.1099	0.1301	0.1201	0.1037	0.08659
1	0.1906	0.2127	0.2175	0.2325	0.2232	0.1732	0.1043	0.1053	0.1114	0.1242	0.1157	0.1098
10	0.1862	0.2258	0.2289	0.2447	0.2197	0.1686	0.08311	0.1148	0.1195	0.1363	0.0962	0.08262
100	0.2306	0.3059	0.2731	0.2894	0.241	0.2282	0.1099	0.1401	0.1068	0.1093	0.08689	0.09971

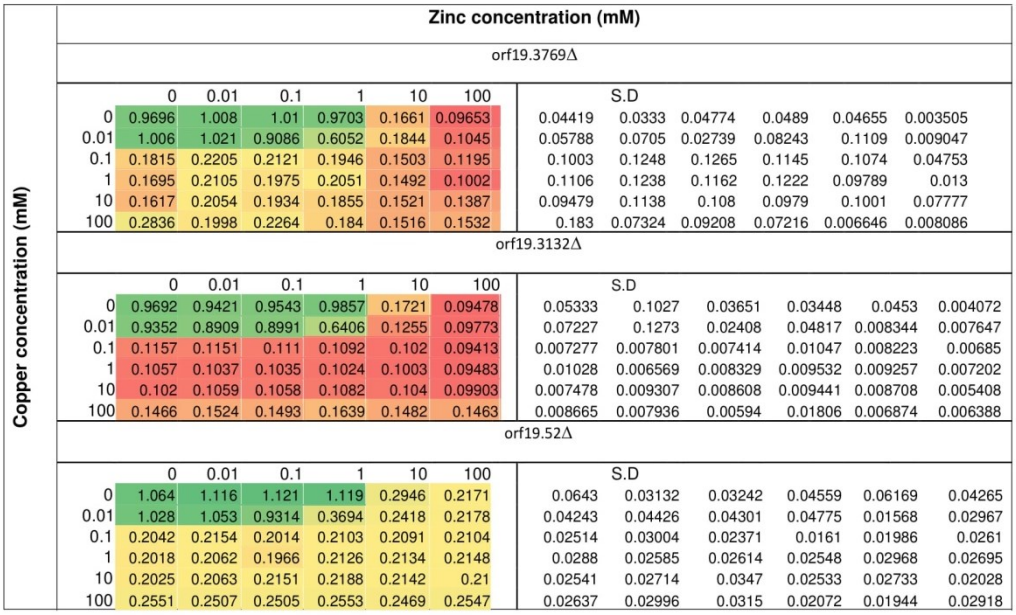
*zrc1*Δ

concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	1.008	0.8503	0.2348	0.1038	0.09928	0.0917	0.01776	0.02804	0.08196	0.01215	0.0105	0.00303
0.01	1.033	0.975	0.3186	0.1341	0.1362	0.0933	0.09865	0.09448	0.1166	0.06108	0.08415	0.00211
0.1	0.1349	0.1647	0.1508	0.1277	0.1289	0.0985	0.06825	0.0795	0.06692	0.06954	0.07073	0.01071
1	0.1286	0.1666	0.1805	0.1503	0.1387	0.1046	0.07137	0.08771	0.1004	0.07265	0.08919	0.02228
10	0.1367	0.149	0.1488	0.1302	0.1253	0.09608	0.07911	0.08134	0.05911	0.06375	0.05626	0.00015
100	0.1415	0.1429	0.1425	0.15	0.2577	0.1421	0.002893	0.00352	0.0002828	0.01112	0.2294	0.002113

orf19.3874Δ

	0	0.01	0.1		10	100	S.D					
0	1.059	1.074	1.012	1.05	0.2859	0.1448	0.1591	0.1704	0.0935	0.1351	0.1807	0.06339
0.01	1.021	0.9701	0.9507	0.7958	0.2135	0.1522	0.05391	0.1001	0.07488	0.1729	0.09477	0.07211
0.1	0.1406	0.149	0.1657	0.1562	0.1628	0.1551	0.05751	0.07905	0.05189	0.06484	0.07816	0.06129
1	0.155	0.1666	0.1476	0.1536	0.1576	0.1493	0.06837	0.07593	0.05887	0.06806	0.07064	0.0661
10	0.1522	0.1602	0.1561	0.1528	0.1567	0.1614	0.06167	0.07103	0.06279	0.05779	0.06629	0.07247
100	0.2539	0.2497	0.2147	0.2597	0.1993	0.2128	0.1473	0.1262	0.07687	0.1384	0.0641	0.08261

Cont.



Zinc concentration (mM)

orf19.3769Δ

Copper concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9696	1.008	1.01	0.9703	0.1661	0.09653	0.04419	0.0333	0.04774	0.0489	0.04655	0.003505
0.01	1.006	1.021	0.9086	0.6052	0.1844	0.1045	0.05788	0.0705	0.02739	0.08243	0.1109	0.009047
0.1	0.1815	0.2205	0.2121	0.1946	0.1503	0.1195	0.1003	0.1248	0.1265	0.1145	0.1074	0.04753
1	0.1695	0.2105	0.1975	0.2051	0.1492	0.1002	0.1106	0.1238	0.1162	0.1222	0.09789	0.013
10	0.1617	0.2054	0.1934	0.1855	0.1521	0.1387	0.09479	0.1138	0.108	0.0979	0.1001	0.07777
100	0.2836	0.1998	0.2264	0.184	0.1516	0.1532	0.183	0.07324	0.09208	0.07216	0.006646	0.008086

orf19.3132Δ

	0	0.01	0.1	1	10	100	S.D					
0	0.9692	0.9421	0.9543	0.9857	0.1721	0.09478	0.05333	0.1027	0.03651	0.03448	0.0453	0.004072
0.01	0.9352	0.8909	0.8991	0.6406	0.1255	0.09773	0.07227	0.1273	0.02408	0.04817	0.008344	0.007647
0.1	0.1157	0.1151	0.111	0.1092	0.102	0.09413	0.007277	0.007801	0.007414	0.01047	0.008223	0.00685
1	0.1057	0.1037	0.1035	0.1024	0.1003	0.09483	0.01028	0.006569	0.008329	0.009532	0.009257	0.007202
10	0.102	0.1059	0.1058	0.1082	0.104	0.09903	0.007478	0.009307	0.008608	0.009441	0.008708	0.005408
100	0.1466	0.1524	0.1493	0.1639	0.1482	0.1463	0.008665	0.007936	0.00594	0.01806	0.006874	0.006388

orf19.52Δ

	0	0.01	0.1	1	10	100	S.D					
0	1.064	1.116	1.121	1.119	0.2946	0.2171	0.0643	0.03132	0.03242	0.04559	0.06169	0.04265
0.01	1.028	1.053	0.9314	0.3694	0.2418	0.2178	0.04243	0.04426	0.04301	0.04775	0.01568	0.02967
0.1	0.2042	0.2154	0.2014	0.2103	0.2091	0.2104	0.02514	0.03004	0.02371	0.0161	0.01986	0.0261
1	0.2018	0.2062	0.1966	0.2126	0.2134	0.2148	0.0288	0.02585	0.02614	0.02548	0.02968	0.02695
10	0.2025	0.2063	0.2151	0.2188	0.2142	0.21	0.02541	0.02714	0.0347	0.02533	0.02733	0.02028
100	0.2551	0.2507	0.2505	0.2553	0.2469	0.2547	0.02637	0.02996	0.0315	0.02072	0.01944	0.02918

Zinc concentration (mM)

Wild-type

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9362	0.9519	0.9378	0.9313	0.1717	0.09655	0.01806	0.02081	0.0114	0.02266	0.02006	0.00243
0.01	0.9221	0.9811	0.9856	0.9488	0.1663	0.097	0.03993	0.02386	0.03617	0.02727	0.05407	0.006373
0.1	0.8747	0.9429	0.9307	0.9659	0.2277	0.1447	0.09798	0.07033	0.1016	0.1277	0.1075	0.05884
1	0.7422	0.8107	0.6618	0.8511	0.2965	0.2314	0.197	0.09041	0.0929	0.1181	0.094	0.08278
100	0.1103	0.1118	0.1149	0.109	0.1084	0.1114	0.004664	0.003761	0.006289	0.002982	0.004117	0.003634

*zrc1*Δ

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9451	0.8249	0.2471	0.1023	0.09098	0.09135	0.02097	0.05579	0.04713	0.006878	0.002799	0.002901
0.01	0.9252	0.8312	0.2821	0.1071	0.094	0.09235	0.01708	0.03721	0.0284	0.005805	0.001309	0.002373
0.1	0.8021	0.8568	0.5418	0.169	0.1189	0.105	0.03176	0.05983	0.1123	0.07488	0.03502	0.009892
1	0.7772	0.7858	0.6174	0.24	0.2417	0.1827	0.09619	0.07159	0.07552	0.06887	0.09292	0.03016
100	0.109	0.1069	0.1079	0.109	0.1106	0.1121	0.002503	0.001348	0.001269	0.001455	0.004152	0.002187

orf19.3874Δ

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9113	0.9194	0.9406	0.9103	0.2239	0.1028	0.01157	0.02319	0.03142	0.03248	0.04732	0.01006
0.01	0.8851	0.8892	0.8881	0.8739	0.2053	0.1064	0.03105	0.03841	0.02761	0.05614	0.04618	0.01135
0.1	0.7852	0.765	0.8183	0.8438	0.1912	0.1199	0.03992	0.02809	0.02338	0.02693	0.04398	0.02122
1	0.5506	0.6192	0.5319	0.6787	0.2604	0.199	0.0719	0.0604	0.09056	0.02205	0.04786	0.01514
100	0.1258	0.1253	0.1297	0.1309	0.1261	0.1228	0.01911	0.006891	0.00789	0.007691	0.008335	0.01268

Cont.

Zinc concentration (mM)

orf19.3769Δ

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9511	0.9554	0.9639	0.9444	0.2234	0.09795	0.03522	0.04938	0.04768	0.0645	0.04662	0.00894
0.01	0.9413	0.953	0.9608	0.924	0.1837	0.1026	0.04986	0.03927	0.03285	0.03423	0.01002	0.01725
0.1	0.8339	0.8527	0.8719	0.9114	0.193	0.111	0.03837	0.01119	0.0118	0.04039	0.01755	0.01602
1	0.6237	0.6949	0.6172	0.8053	0.2786	0.1859	0.1003	0.009269	0.09135	0.0221	0.03975	0.01819
100	0.1234	0.133	0.1327	0.136	0.1273	0.1226	0.01875	0.02143	0.01928	0.02045	0.01645	0.01854

orf19.3132Δ

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	0.9418	0.8876	1.028	0.9514	0.2374	0.1178	0.02519	0.1189	0.1525	0.0404	0.0843	0.02148
0.01	0.9545	0.9391	0.9565	0.945	0.2094	0.1309	0.02233	0.0151	0.03878	0.0353	0.009045	0.03332
0.1	0.8561	0.8607	0.8781	0.8598	0.2332	0.1418	0.01415	0.02952	0.01116	0.04777	0.04518	0.0329
1	0.6285	0.6725	0.6014	0.7458	0.3139	0.213	0.02614	0.04067	0.01965	0.07369	0.0494	0.03976
100	0.1746	0.1748	0.1836	0.1939	0.1953	0.1671	0.03424	0.01687	0.007687	0.02181	0.02246	0.04922

orf19.52Δ

iron concentration (mM)	0	0.01	0.1	1	10	100	S.D					
0	1.043	1.008	1.001	0.996	0.3135	0.1596	0.06436	0.09258	0.1167	0.08266	0.0791	0.1116
0.01	0.9607	0.989	0.9378	0.9798	0.2474	0.1365	0.05506	0.09591	0.03598	0.06924	0.0287	0.04084
0.1	0.8593	0.8578	0.8823	0.9038	0.279	0.1474	0.04794	0.07683	0.07279	0.06107	0.03553	0.01762
1	0.6903	0.722	0.6376	0.826	0.3728	0.2344	0.03864	0.1499	0.07675	0.1312	0.0574	0.03122
100	0.1245	0.1256	0.1552	0.154	0.151	0.1382	0.02492	0.02123	0.04418	0.02537	0.01924	0.02227

Figure S8

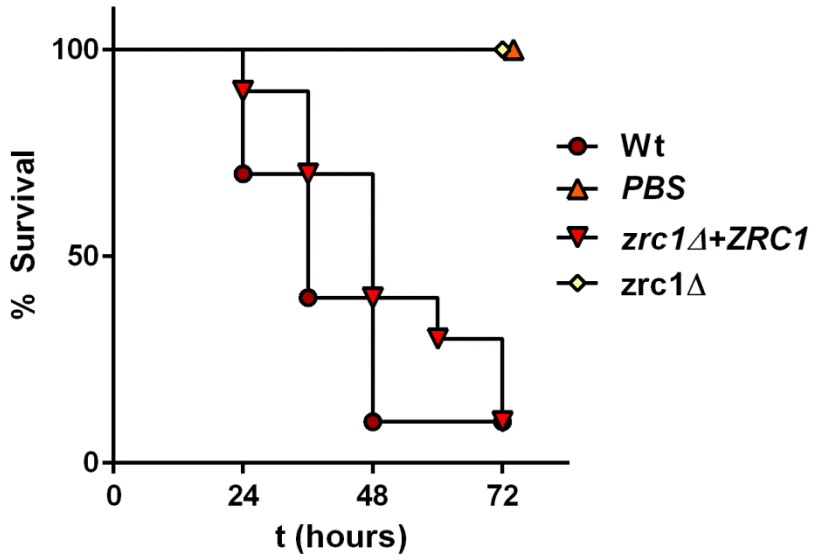
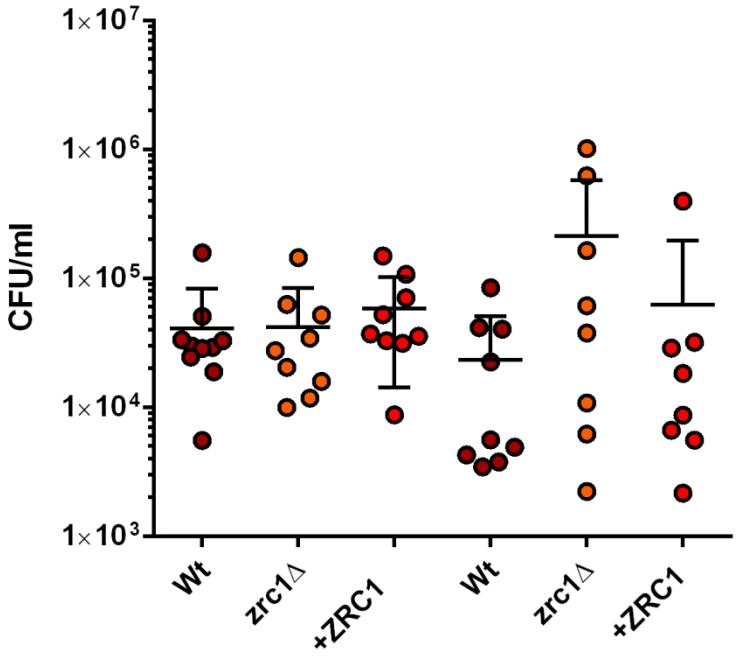


Figure S9



4.3. Manuscript III: Skrahina *et al*., in preparation, 2018

Combined transcriptomics and transcription factor functional analysis of *Candida albicans* in response to zinc limitation

Volha Skrahina, Duncan Wilson, Bernhard Hube, Sascha Brunke

In preparation for Metallomics.

Summary:

Zinc is an essential trace metal for all living organisms. During infections the host controls access to essential metals, including zinc, *via* nutritional immunity processes, and the pathogen applies various strategies to obtain the vital metal from the host. *Candida albicans* is both a fungal member of the human microbiota and an opportunistic pathogen, which can cause infections at multiple body sites. Thus, the fungus needs to sense and acquire zinc in various niches that differ greatly in the metal availability. So far, the response to zinc starvation has not been investigated in detail in *C. albicans*, as is the case for *S. cerevisiae*. Therefore, we analyzed the transcriptional response of *C. albicans* to zinc deprivation *in vitro* using wild type and *zrt2*Δ/Δ mutant, lacking a plasma membrane zinc importer, strains. The wild type and *zrt2*Δ/Δ transcriptional data demonstrated that processes, such as zinc homeostasis, oxidative stress, transcription, translation, metabolism, and biosynthesis are affected by zinc availability. In contrast to the wild type, the *zrt2*Δ/Δ mutant activated zinc uptake machinery genes (even in the presence of zinc) and, under low zinc levels, was unable to regulate both the adaptor protein Ste50 (involved in MAPK signaling pathways) and the ubiquitin-proteasome system.

Two transcription factors regulate the expression of zinc homeostasis genes in *C. albicans*: Csr1 and the more recently described Sut1. In order to identify additional transcriptional factors involved in zinc homeostasis, we screened a transcription factor deletion library under low zinc levels. We identified transcriptional factors that are potential candidates for regulating zinc starvation response. For example, *ssn6*Δ/Δ, detected in the library screen, was found to be unable to activate zinc uptake genes in response to zinc deficiency. Thus, the presence of Ssn6, along with previously described transcriptional factors, is required for zinc limitation response in *C. albicans*.

To our knowledge, this is the first comprehensive study describing zinc homeostasis on both transcriptional and phenotypic levels in *C. albicans*.

Own Contribution:

Volha Skrahina designed the study, planned and carried out experiments, interpreted results (including bioinformatics analyses using GeneSpring GX software package), prepared figures, and wrote the manuscript. The following experiments were performed

in the laboratory: transcriptional profiling using DNA microarrays, qRT-PCRs, and phenotypic analyses. The other authors designed the project, evaluated results, and revised the manuscript.

Estimated authors' contributions:

Volha Skrahina	**55%**
Duncan Wilson	15%
Bernhard Hube	5%
Sascha Brunke	25%

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Prof. Bernhard Hube

Combined transcriptomics and transcription factor functional analysis of *Candida albicans* response to zinc limitation

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Running title: *C. albicans* and zinc starvation response

Key words: zinc limitation, transcriptional response, mutant collection, phenotypic profiling, ROS

Abstract

Zinc is indispensable for all living organisms as a structural and catalytic cofactor for numerous proteins. The availability of zinc is recognized as a central factor in infections, and is at the frontline between limitation by the host, known as nutritional immunity, and sophisticated strategies of pathogens to obtain this essential metal from the host. Zinc-dependent regulation in the opportunistic pathogen *Candida albicans*, a common member of the microbiota in healthy humans, has not been investigated in detail so far. Here, we characterize *in vitro* the transcriptional response to zinc deprivation of *C. albicans* wild type and *zrt2*Δ/Δ, a mutant lacking a plasma membrane zinc transporter. Critical cellular processes, such as transcription, translation, metabolism, and biosynthesis were affected by zinc limitation. Furthermore, we screened a transcription factor deletion library under low zinc levels and found a small number of mutants lacking factors with potential roles in zinc homeostasis regulation. Among them, the *ssn6*Δ/Δ mutant showed a zinc-dependent growth defect, which seems to be based on its inability to fully upregulate its zinc uptake machinery. This suggests Ssn6 to be a novel factor in the regulation of zinc homeostasis in *C. albicans*.

Introduction

Zinc is a structural and catalytic co-factor for a broad range of proteins, and approximately 9 % of eukaryotic proteins require zinc for their function (Andreini, Bertini et al. 2009). The availability of zinc is a central factor in infections: The host is able to restrict microbes' access to zinc in order to prevent microbial replication in a process known as "nutritional immunity"(Weinberg 1975) and in turn, pathogens employ various strategies to obtain zinc from the host environment (Hood and Skaar 2012). For example, the host can limit free metals from the pathogens *via* histatins, antimicrobial peptides in saliva that bind zinc and copper (Gusman, Lendenmann et al. 2001), and calprotectin, a cytosolic protein of neutrophils that binds zinc, manganese, iron, nickel, and copper (Nakashige, Zhang et al. 2015, Kelliher and Kehl-Fie 2016, Baker, Nakashige et al. 2017, Besold, Gilston et al. 2017, Nakashige, Zygiel et al. 2017). Calprotectin is mainly released during the formation of neutrophil extracellular traps (Clohessy and Golden 1995, Gebhardt, Nemeth et al. 2006, Fuchs, Abed et al. 2007, Urban, Ermert et al. 2009) and its ability to sequester metals effectively inhibits bacterial and fungal growth (Corbin, Seeley et al. 2008, Hood, Mortensen et al. 2012, Kehl-Fie, Zhang et al. 2013, Amich, Vicentefranqueira et al. 2014, Gaddy, Radin et al. 2015, Clark, Jhingran et al. 2016, Juttukonda, Chazin et al. 2016). Additionally, many host niches have a low metal solubility due to their generally neutral pH, and thus metal accessibility for the pathogens is low. In the opposite extreme, zinc excess can also be toxic to cells, due to the competition with other metals for protein metal-binding sites (McDevitt, Ogunniyi et al. 2011, Gu and Imlay 2013). For example, vertebrates are known to increase intraphagosomal zinc levels to cause zinc toxicity for phagocytosed *Mycobacterium tuberculosis* (Botella, Peyron et al. 2011). As both limitation and excess of zinc are harmful, a tight regulation of zinc homeostasis is essential for pathogenic microorganisms.

Candida albicans is an opportunistic fungal pathogen that infects millions of people every year, with an estimated number of 400,000 cases of life-threatening infections annually, which are associated with a mortality rate of 46-75%, even with antifungal therapy available (Brown, Denning et al. 2012, Jabra-Rizk, Kong et al. 2016). As a commensal and opportunistic pathogen, *C. albicans* is able to cope with zinc limitation: *C. albicans* assimilates zinc from its extracellular environment *via* the plasma membrane zinc importers Zrt1 and Zrt2, and by a "zincophore" system, consisting of Pra1 and Zrt1 (Citiulo, Jacobsen et al. 2012, Crawford, Lehtovirta-Morley et al. 2018). Pra1 is secreted into the extracellular space in response to alkaline pH and low zinc levels, where it binds with high affinity zinc ions and delivers zinc to the plasma membrane zinc importer Zrt1 (Citiulo, Jacobsen et al. 2012). In fungi, vacuoles and

zincosomes serve for zinc sequestration, storage, and detoxification (MacDiarmid, Gaither et al. 2000, Devirgiliis, Murgia et al. 2004, Cho, Hu et al. 2018, Crawford, Lehtovirta-Morley et al. 2018). *C. albicans* can cope with high zinc levels by accumulating zinc in zincosomes *via* the Zrc1 importer (Crawford, Lehtovirta-Morley et al. 2018). In *S. cerevisiae* the mobilization of zinc from the vacuole occurs *via* the vacuolar zinc exporter Zrt3 (MacDiarmid, Gaither et al. 2000), of which *C. albicans* contains an uncharacterized ortholog, orf19.1534 (*ZRT3*). *C. albicans* zinc effectors are positively regulated by transcriptional factors (TFs) Csr1, an ortholog of *S. cerevisiae* Zap1 (zinc responsive activator protein 1)(Nobile, Nett et al. 2009), and Sut1 (Xu, Solis et al. 2015).

Within fungi, zinc homeostasis has been investigated in detail in *S. cerevisiae*: There are more than 400 genes essential for growth under low zinc levels, which include genes directly involved in zinc homeostasis, chromatin modification, endoplasmic reticulum function, protein folding, vesicular trafficking, and oxidative stress resistance (North, Steffen et al. 2012). In this study, we characterize the response to zinc limitation in the pathogen *C. albicans*. To do so, we analyzed the transcriptional response to low zinc levels in wild type and *zrt2*Δ/Δ strains and screened a transcription factor deletion library under low zinc levels to define TFs essential for zinc homeostasis.

Materials and Methods

Strains and culture conditions

The *C. albicans* strains used within this study are listed in the **Table S1.** The triple-auxotrophic strain BWP17 complemented with plasmid CIp30 serves as the isogenic wild type control (BWP17+CIp30). Homozygous *C. albicans* mutants were constructed as previously described (Walther and Wendland 2003, Mayer, Wilson et al. 2012) and the primers used in the study are listed in the **Table S2**.

A *C. albicans* transcription factor deletion library was used for the phenotypical screening under zinc limitation (Homann, Dea et al. 2009). Wild type and mutant strains from Homann *et al.* are arginine auxotrophs, and all media used for the experiments with these strains was supplemented with arginine (20 µg mL^{-1}).

C. albicans strains were routinely grown in YPD complex medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C with shaking at 180 rpm. To perform zinc limitation conditions, after YPD pre-culture, strains were washed three times in nano pure water and further grown in plastic universal flasks (pre-treated with 3,7% HCl, and washed with nano pure water). For zinc limitation experiments, *C. albicans* cells were grown in limited zinc medium (LZM) (the components are listed in the **Table S3**), in SD without zinc (0.69% yeast nitrogen base without zinc [Formedium], 2% glucose), or in SD without zinc with 1mM EDTA. For iron limitation experiments, *C. albicans* cells were grown in limited iron medium (LIM) (the components are listed in the **Table S3**).

Transcriptional profiling

For the zinc limitation time series experiment, the washed wild type cells were set to $5X10^6$ cells/mL in LZM+5µM $ZnSO_4$ for the adaptation phase, and after 4 h, the sample, designated 0 min, was taken. The rest of the cells were washed, inoculated in LZM for the limitation phase, and samples were taken after 30, 60, 120, 240, 480, 960, and 1440 minutes.

For zinc limitation and refeeding experiments, washed wild type and *zrt*2Δ/Δ strains were inoculated at OD=0,1 in LZM for the limitation phase, and after 24 h, samples were taken. The rest of the cells were washed and inoculated at OD=0,1 in LZM+300µM zinc citrate for the refeeding phase, and after 24 h the samples were taken.

For the investigation of the transcriptional response in a defined media, washed wild type and *zrt*2Δ/Δ strains were inoculated at OD=0,1 in LZM+5µM $ZnSO_4$ and samples were taken after 4 h and 16 h.

The samples were collected at the indicated time points by centrifugation. Samples were immediately used for RNA extraction or frozen in liquid Nitrogen and stored at -80°C until the use. For RNA isolation, the RNeasy kit [Qiagen] was used, according to the manufacturer's instructions. RNA quality was determined using a BioAnalyzer instrument [Agilent], and samples were labeled using the Quick Amp Labeling Kit [Agilent] with Cy5-CTP [GE Healthcare]. Samples were collected in biological triplets at all indicated time points.

Microarray Analyses

Microarrays were performed with Cy5-CTP labeled cRNA [GE Healthcare] (obtained from the RNA sampled at the indicated time points) and Cy3-CTP labeled cRNA [GE Healthcare], which was used as a common reference (obtained from the RNA sampled from an exponentially growing *C. albicans* cells in YPD). Hybridizations, scanning, and data analyses were performed as previously described (Ramachandra, Linde et al. 2014). The data was LOWESS normalized and evaluated using the GeneSpring GX software package, version 12.1 [Agilent]. Gene Ontology (GO) Term analyses were performed with the same program, based on the genome and annotations from the Candida Genome Database (Inglis, Arnaud et al. 2012). Gene transcription data will be deposited at the ArrayExpress database prior to the publication of the manuscript.

To analyze the zinc limitation time series experiment transcriptional data set of the wild type, each time point was compared to the 0 time point (4 h in LZM+5µM $ZnSO_4$) and statistically significant genes were selected (T-Test Unpaired, P > 0.05; Fold Change > 2.0).

To analyze the transcriptional response of the wild type and *zrt2*Δ/Δ in LZM+5µM $ZnSO_4$, LZM, and LZM+300µM zinc citrate the following data sets were received after statistical analyses (T-Test Unpaired, P > 0.05; Fold Change > 2.0): 16 h in LZM+5µM $ZnSO_4$, 24 h in LZM, and 24 h in LZM+300µM zinc citrate and these gene groups were analyzed.

Transcription factor deletion library screening

Phenotypic profiling was performed with the *C. albicans* transcription factor deletion library (Homann, Dea et al. 2009) as described in the **Figure S1**. The growth experiments were performed in 96-well plates [TPP plates, Sigma-Aldrich]. The stains were pre-cultured in YPD for 48 h. Afterwards the cells were washed three times in nano pure water and inoculated at OD=0,05 in LZM for the starvation phase. The OD was measured after 24 h (further called "end OD"), which was followed by the washing step and inoculation of the cells at OD=0,005 in LZM (for the limitation phase), in

LZM+5µM$ZnSO_4$ and in LZM+5µM zinc citrate (for the refeeding phase) and after 24 h the end OD was measured. The OD was always measured after setting the cells to the initial OD to know the exact starting OD (further called “initial OD”) and to check for pipetting errors. The experiment was performed in biological duplicates.

The data were analyzed as described in the formula below.

$$x = \frac{\text{end OD mutant} - \text{initial OD mutant}}{\text{end OD wild type} - \text{initial OD wild type}}$$

x – Relative growth of the strains

Growth curves analyses

For phenotypic profiling, strains were grown in YPD overnight, washed three times in nano pure water and inoculated at OD=0,005 in zinc limited media (media types are described in the “strains and culture conditions*”* section) for the starvation phase for 24 h in order to largely deplete internal zinc storage. Afterwards, the cells were washed again in nano pure water and inoculated at OD=0,005 in zinc limited media and in media with the addition of zinc. During this time, the cells were placed in the reader [TECAN infinite M200Pro], grown at 30°C, and OD 600 was determined after 30 s of orbital shaking every 30 min.

Quantitative Real-Time Reverse Transcription-PCR (qRT-PCR)

Wild type (BWP17+CIp30), *ssn6*Δ/Δ, and *ssn6*Δ/Δ+*SSN6* strains, after YPD pre-culture, were washed three times in nano pure water and inoculated at OD=0,1 in YPD, LZM+300µM $ZnSO_4$,and LZM; and in YPD, SD+25µM $ZnSO_4$, and in SD without zinc. After 4 h, the cells were frozen in liquid nitrogen, followed by a total RNA extraction [Qiagen RNeasy]. The total RNA was treated with DNase [Epicenter Baseline-ZERO] and purified using the kit [Qiagen RNeasy]. RNA quality was verified *via* the bioanalyzer instrument [Agilent]. The RNA concentration was determined using a NanoQuant plate in the reader [TECAN infinite M200Pro]. For each sample, 500 ng RNA was transcribed into cDNA, which was checked *via* PCR for genomic DNA (gDNA) contamination, using intron-spanning primers (**Table S2**). A total amount of 1,85 ng cDNA was used for each qRT-PCR using the fluorescent dye EvaGreen [Bio&Sell] in the thermal cycler [CFX96™ Real-Time System Bio-Rad] in biological triplicates. Expression rates were determined relative to the housekeeping gene *ACT1* and analyzed using the Software [Bio-Rad CFX Manager]. All primers are listed in the **Table S2**. Statistical analyses were performed by one way ANOVA, followed by Dunnet tests, where the mean of each mutant strain was compared with the mean of the wild type strain.

Results and discussion

Transcriptional response of Candida albicans to zinc limitation

To analyze the transcriptional response of the *C. albicans* wild type to low zinc levels, a time series experiment under zinc limitation was performed. After an adaptation phase of 4 hours in LZM with sufficient zinc (LZM+5µM $ZnSO_4$), the wild type was transferred into zinc-free LZM for the starvation phase. Samples were taken at 0, 30, 60, 120, 240, 480, 960, and 1440 min for the transcriptional analysis. The adaptation step allowed us to discriminate zinc-dependent transcriptional changes from those resulting from the medium change alone.

Our data show that zinc limitation is detected by fungal cells, as genes for zinc transporters (*ZRT1*, *ZRT2*, *ZRT3*, and orf19.1536 (called *ZRC1* in the following)) as well as for the zincophore (*PRA1*) display zinc-dependent transcript level changes: The expression of the uptake system for extracellular zinc (genes *ZRT1*, *ZRT2*, and *PRA1*) was upregulated mainly at later time points (**Figure 1 ABC**). Additionally, zinc mobilization from internal storage seems to occur, as *ZRT3,* an orthologue of a *S. cerevisiae* zinc vacuole exporter gene, was induced and expression of *ZRC1*, a zincosome zinc importer gene*,* was decreased in LZM (**Figure 1 DE**).

PRA1 and *ZRT1* share the same upstream intergenic region and are known to be co-expressed under low zinc levels solely at alkaline conditions (Sentandreu, Elorza et al. 1998, Ihmels, Bergmann et al. 2005, Citiulo, Jacobsen et al. 2012). Interestingly, we observed an upregulation of transcript levels of both *ZRT1* and *PRA1* in response to low zinc levels (**Figure 1 AC**), although our zinc limitation medium was acidic (LZM, pH 4.6). We thus compared the phenotypes of *zrt2*Δ/Δ and *zrt1*Δ/Δ in our assay conditions with the literature. The expression level of the second plasma membrane zinc importer gene *ZRT2* is known to be upregulated under zinc limitation independent of pH levels (Crawford, Lehtovirta-Morley et al. 2018). Accordingly, a *C. albicans zrt1*Δ/Δ strain is known to grow robustly under zinc limitation at both acidic and alkaline conditions, as *ZRT2* is expressed at acidic pH. In contrast, a *zrt2*Δ/Δ strain shows a growth defect under zinc limitation and acidic pH, as the compensatory *ZRT1* expression was so far only detected at alkaline pH (Crawford, Lehtovirta-Morley et al. 2018). Against our transcriptome data, but in agreement with previous literature (Crawford, Lehtovirta-Morley et al. 2018), we also observed a growth defect of *zrt2*Δ/Δ under zinc limitation only at acidic pH (data not shown). These differences may be explained by the different methods used to detect expression of *ZRT1*, as in the previous work fluorescence levels of a *ZRT1* promoter-driven *GFP* were detected rather than *ZRT1* mRNA levels. Thus, our observed transcription of *ZRT1* at acidic

conditions may not result in a functional Zrt1 protein. Interestingly, Pra1 was shown to have the highest zinc binding capacity at neutral/alkaline pH (Loboda and Rowinska-Zyrek 2017), but was recently shown to be transcriptionally induced in acidic conditions as well (Malavia, Lehtovirta-Morley et al. 2017).

So far, the induction of the vacuolar zinc exporter, *ZRT3,* and its orthologues has been described for *S. cerevisiae, B. dermatitidis*, and *C. dubliniensis* under zinc limitation (MacDiarmid, Gaither et al. 2000, Bottcher, Palige et al. 2015, Munoz, Gauthier et al. 2015). The zinc limitation-dependent upregulation of the *ZRT3* orthologue of *C. albicans* (**Figure 1 D**) and its sequence similarity with *S. cerevisiae ZRT3* indicates that Zrt3 is likely to act as a vacuolar zinc exporter in *C. albicans* as well.

Using a global approach *via* hierarchical clustering, two main clusters were identified in the transcriptome data: an early cluster (30 min, 60 min, 120 min, 240 min, and, surprisingly, 1440 min) with the highest similarity between 120 min and 240 min, and a late cluster (480 min and 960 min) with the highest similarity between their entities (**Figure S2**).

Representative genes for early and late clusters were selected based on their consistent up- or downregulation at both 120 and 240 min and both at 480 and 960 min, respectively (**Figure S3 ABCD**). These gene groups were then functionally analyzed based on Gene Ontology (GO) term enrichment (**Figure 2, Table S4** and **Table S5**).

Certain GO terms from upregulated genes were found enriched at both early (120-240 min) and late (480-960 min) phases, including oxidative stress resistance (**Figure 2 AC**). Indeed the transcription of genes encoding superoxide dismutases (*SOD1* and *SOD2*) was significantly induced in both stages. From *S. cerevisiae* (Wu, Bird et al. 2007, Wu, Roje et al. 2009, Wu, Steffen et al. 2009) it is known that zinc deficiency can lead to an intracellular accumulation of reactive oxygen species (ROS) (Eide 2011). Moreover, the presence of Sod1 activity in *S. cerevisiae* was found to be critical for growth under low zinc levels (Wu, Steffen et al. 2009). According to the transcriptional data, *C. albicans* also experiences increased ROS levels, as it upregulates oxidative stress defense genes.

The GO term “cellular zinc ion homeostasis” was found significantly upregulated at 480-960 min (**Figure 2 C**), which supports the late transcriptional response of zinc uptake and mobilization machinery, as indicated in **Figure 1.** Likely, internal zinc storage was sufficiently high to avoid zinc starvation stress in the early phase of the experiment. Interestingly, the transcript levels of copper transporter genes (*CTR1*,

SCO1) and the hemin and hemoglobin uptake gene (*PGA10*) (that was shown to be upregulated under iron starvation) (Weissman and Kornitzer 2004) were significantly reduced at 480-960 min, indicating a zinc-specific starvation signature of the cells.

Early and late phase downregulated genes were found enriched for the reduction of translation and ribosome biogenesis (**Figure 2 BD**), which includes the downregulation of genes encoding the ribosomal structural subunits (*RPL12*, *RPL5*, *RPL18*, and *RPL17B*) at 120-240 min and (*RPS23A*, *RPS21B*, *RPL32*, and *RPL23A*) at 480-960 min. Additionally, the transcript levels of genes encoding histone subunits (*HTA3*, *HHF1*), non-histone chromatin component (*NHP6A*), and histone H2A phosphatase (*PHO15*) were significantly reduced. Moreover, we observed the reduction of metabolic processes under zinc limitation at both 120-240 min and 480-960 min time points. These included the downregulation of biosynthetic processes (**Figure 2 BD**) such as synthesis of asparagine (*ASN1*), methionine (*MET2*, *MET6*), aromatic acids (*ARO4*), riboflavin (*RIB3*), ergosterol (*ERG1*, *ERG6*, *ERG13*, and *ERG251*), and fatty acids (*FAS1*, *FAS2*). Overall, the downregulation of transcription and translation, which leads to the general reduction of metabolism in *C. albicans*, is a common characteristic of zinc limitation responses in prokaryotes and eukaryotes (Mazus, Falchuk et al. 1984, North, Steffen et al. 2012, Neupane, Jacquez et al. 2017).

There are only three genes (*SOD1*, *HSP70*, and *EEP1*) that were significantly upregulated at 120 min and stayed induced until 1440 min and only one gene (*ASN1*) that was significantly downregulated at 240 min and the expression remained reduced until 1440 min (**Figure 3**). Interestingly, in *C. albicans, EEP1* codes for a protein with a predicted endonuclease/exonuclease/phosphatase family domain, which is mainly found in magnesium-dependent endonucleases and phosphatases (Dlakic 2000). Additionally, the presence of Hsp70, a heat-shock protein (also called Ssa1), was shown to be required for virulence in *C. albicans* (Sun, Solis et al. 2010). So far, the role of these four genes in mediating zinc limitation stress resistance is unknown, but the fact that these genes remain significantly regulated within the experiment suggests that they have an important role.

Transcriptional response of the wild type and zrt2Δ/Δ under zinc limitation and refeeding conditions

Having established the zinc starvation response of the wild type, we continued to investigate the function of Zrt2 in zinc homeostasis in *C. albicans*. We initially obtained the transcriptome of the *zrt2*Δ/Δ mutant after 4 h and 16 h in LZM+5µM $ZnSO_4$. In contrast to the wild type (**Figure 4 A1B1C1**), we found that expression of *ZRT1, PRA1*, and *ZRT3* was induced at 16h in the mutant (**Figure 4 A2B2C2**), even in

the presence of 5µM $ZnS0_4$. Thus, the *zrt2*Δ/Δ strain experiences and senses zinc starvation in our original reference medium.

Given these differences in the threshold for zinc starvation, we followed a different approach to directly compare the zinc-dependent transcriptome of wild type and *zrt2*Δ/Δ strains. First, we determined a zinc concentration that supports the growth of both the wild type and *zrt2*Δ/Δ mutant. To do so, we depleted the internal zinc storage by growing the cells for 24h in LZM, followed by a change to LZM containing various concentrations of zinc (not shown). It was found that after LZM-based starvation, the wild type was able to grow in both LZM+300µM zinc citrate and in LZM+300µM $ZnSO_4$ (**Figure 5 A**), while the *zrt2*Δ/Δ mutant was not able to grow in LZM+300µM $ZnSO_4$. LZM+300µM zinc citrate supported the growth of the *zrt2*Δ/Δ strain similar to the wild type (**Figure 5 B**) and therefore this condition was used in the following experiments.

We then established a common base for both wild type and mutant strains by sampling after 24 h starvation in LZM without Zn to induce upregulation of genes essential under zinc limitation. The cultures were then transferred to LZM+300µM zinc citrate and sampled again after 24 h. Indeed, zinc homeostasis genes possessed a zinc content-dependent expression pattern (**Figure 4**). Further we combined these datasets with the transcriptome of the wild type and *zrt2*Δ/Δ mutant grown in LZM+5µM $ZnSO_4$. Thus, we defined zinc-responsive genes as those that were upregulated in LZM without zinc and downregulated in LZM+300µM zinc citrate (unpaired t-test, $p > 0.05$; fold change > 2.0) and *vice versa* (**Figure S4**). These groups of genes were then functionally analyzed based on GO terms enrichment (**Figure S5**, **Table S6** and **Table S7**). Specific for *zrt2*Δ/Δ "response to oxidative stress" was found enriched, which includes *SOD1*, *TSA1,* encoding hydroperoxide peroxidase C, *HSP104,* coding for a heat-shock protein, and *ZWF1*, which encodes a glucose-6-phosphate dehydrogenase. The expression of these genes is known to be induced by oxidative stress and both Sod1 and Tsa1 are known to mediate oxidative stress resistance in *C. albicans* (Hwang, Rhie et al. 1999, Urban, Xiong et al. 2005, Enjalbert, Smith et al. 2006).

There are 67 zinc-responsive upregulated genes (**Figure S6 A, Table S8 A**) and 11 zinc-responsive downregulated genes (**Figure S6 B, Table S8 B**) in the wild type that were found not to be significantly regulated in the *zrt2*Δ/Δ mutant, partly because their fold changes did not reach the wild type levels ($p < 0.05$; Fold Change > 2.0). However, 11 upregulated (**Table S8 A**) and 4 downregulated (**Table S8 B**) genes

showed a clear qualitatively different regulation in the *zrt2*Δ/Δ mutant in comparison to the wild type. These genes are represented in **Table 1**.

Zrt1 is a transceptor in *S. cerevisiae* as it both imports zinc ions and mediates activation of the PKA pathway (Schothorst, Zeebroeck et al. 2017). New data indicates that there could be a similar transceptor in *C. albicans*. For example, *STE50* is not regulated in the *zrt2*Δ/Δ mutant under low zinc levels in contrast to the wild type (**Table 1**). Ste50 contributes to the mitogen-activated protein kinase (MAPK) pathway in *C. albicans* (Ramezani-Rad 2003) and cAMP/PKA and MAPK signalling cascades where shown to regulate common processes (Gerits, Kostenko et al. 2008). Thus Zrt2 in *C. albicans* may be involved in extracellular zinc sensing. This is also supported by the transcriptional response being similar to starvation in *zrt2*Δ/Δ, even in the presence of zinc. Zinc is a structural and catalytic factor for proteins and therefore zinc limitation leads to the disruption of the proper protein folding processes. The ubiquitin-proteasome system is activated due to low zinc levels and helps the cells to deal with unfolded proteins (MacDiarmid, Taggart et al. 2016). The expression of *RTT101*, encoding for the cullin subunit of E3 ubiquitin ligase complex (Laplaza, Bostick et al. 2004), and orf19.6722, encoding for an ortholog in *S. cerevisiae* – Rad4 that is required for turnover of ubiquitination proteins, (Li, Yan et al. 2010) are not regulated in *zrt2*Δ/Δ.

In summary, on the transcriptional level, the absence of Zrt2 leads to a global starvation response (even in the presence of zinc); severe oxidative stress, and the disturbance of both signal transduction and unfolded protein responses under zinc limitation.

Phenotypic profiling of a transcription factor deletion library under zinc limitation and zinc refeeding

Given the central role of transcription factors (TFs) in global transcriptional response, combined with their generally low expression levels, we expanded our analysis by phenotypical profiling of a *C. albicans* transcription factor deletion library (Homann, Dea et al. 2009) under zinc limitation. Two cultures without zinc (24 h starvation and 48 h limitation phases) were followed by a final 48 h 2nd limitation phase and zinc refeeding phases in LZM either with 5µM$ZnSO_4$ or with 5µM zinc citrate (**Figure S1**). Mutant strains with a mean relative growth above ±3σ of the wild type value were considered to have either a growth defect or a growth advantage (**Figure 6**).

Certain mutants were excluded due to a general growth defect: *rbf1*Δ/Δ and *nrg1*Δ/Δ showed a severe defect independent of zinc content (**Figure 6** and **Table S9**). A deletion mutant lacking the central TF for zinc homeostasis, *csr1*Δ/Δ (Kim, Kil et al.

2008), showed an expected growth defect in both zinc limitation phases (**Figure 6** and **Table S9**).

Interestingly, the *ace2*Δ/Δ and *swi4*Δ/Δ mutants showed a growth defect (compared to the wild type) only during the initial starvation phase, and in the limitation phases, these mutants' growth was comparable to the wild type (**Table 2 A,** and **Table S9**). The *S. cerevisiae* orthologues of Ace2 and Swi4 are known to have a growth defect under zinc limitation (North, Steffen et al. 2012). In agreement with our data, the *swi4*Δ mutant had a growth defect only after five generations, but not after fifteen generations in zinc limiting conditions in *S. cerevisiae*. Although that strain was originally discarded as a false negative result (North, Steffen et al. 2012), our observation of the same effect in *C. albicans* makes it more likely that Swi4 (and possibly Ace2) are involved in the transcriptional regulation of zinc homeostasis genes. We hypothesize that the absence of these TFs leads to a longer adaptation time under zinc limitation, which is only recovered during the later phase.

We were most interested in those mutants that showed a severe growth defect under low zinc levels, but were able to grow as well as the wild type strain in the presence of either 5µM $ZnSO_4$ or 5µM zinc citrate (**Table 2 A** and **Table S9**). The number of mutants with a growth defect increased from the starvation phase to the initial limitation phase and even further in the second limitation phase. All of the mutants that had a defect during the first limitation phase (*ssn6*Δ/Δ, *csr1*Δ/Δ, *hfl1*Δ/Δ, *zcf16*Δ/Δ, *gzf3*Δ/Δ, *ume7*Δ/Δ, orf19.3625Δ/Δ, and *czf1*Δ/Δ) continued to show growth defects in the second limitation phase (**Table 2 A** and **Table S9**). In contrast, two mutants of uncharacterized TFs, *zcf2*Δ/Δ and *zcf8*Δ/Δ, had a significant advantage in growth in the second limitation phase (**Table 2 B** and **Table S9**).

The highest degree of overlap between *C. albicans* TFs and their orthologues in *S. cerevisiae* with known defect under zinc starvation was found in the second limitation phase. The *tup1*Δ strain (after five generations), *sfl1*Δ and *tye7*Δ strains (after fifteen generations) were previously shown to have a growth defect under low zinc levels in *S. cerevisiae* (North, Steffen et al. 2012). Additionally, *ume6*Δ also showed a growth defect (after five generations) in *S. cerevisiae* (North, Steffen et al. 2012). *C. albicans* has an orthologue of *S. cerevisiae* Ume6 – Ume7 and a *ume7*Δ/Δ mutant of *C. albicans* has a growth defect under zinc limitation.

The role of Ssn6 under zinc limitation

We verified the transcription factor deletion library results using another growth medium (SD without zinc) and independent mutant clones. The *ssn6*Δ/Δ strain was found to have a growth defect under zinc limitation irrespective of the media type and

the mutant clone (not shown). We therefore constructed independent deletion and complemented strains in the BWP17 background and tested their ability to grow under low zinc levels and with 300µM $ZnSO_4$. This new *ssn6*Δ/Δ strain exhibited a growth defect under low zinc levels, which was recovered in the *ssn6*Δ/Δ+*SSN6* strain (**Figure 7**). To check the metal specificity of this growth disadvantage, the strains were also tested under low iron levels, where we observed no growth defect of *ssn6*Δ/Δ (**Figure 8**).

In light of these phenotypes, we hypothesized that Ssn6 regulates zinc homeostasis genes. First we determined the expression of *SSN6* in YPD, LZM+300 µM $ZnSO_4$, and LZM. As expected, *SSN6* mRNA was undetectable in the *ssn6*Δ/Δ mutant (**Figure 9 A**). Although *SSN6* was expressed (**Figure 9 A**) and functional (**Figure 7**) in the *ssn6*Δ+*SSN6* revertant strain, the expression of *SSN6* was lower than in the wild type in LZM (**Figure 9 A**). Given this strong copy number effect, we focused on the expression of zinc transporters (*ZRT1*, *ZRT2*, and *ZRT3*), zincophore (*PRA1*), and the zinc-responsive transcription factor (*CSR1*) in wild type and *ssn6*Δ/Δ strains grown in YPD, LZM+300µM $ZnSO_4$, and LZM for 4 h *via* qRT-PCR (**Figure 9**). All zinc transporter genes in LZM were significantly down-regulated in *ssn6*Δ/Δ (**Figure 9 CEF**), whilst in LZM *PRA1* expression was unchanged (**Figure 9 D**) compared to the wild type. The transcript levels of *CSR1* in *ssn6*Δ/Δ were comparable to wild type, (**Figure 9 B**) this is interesting, because it indicates Ssn6-depedent and Csr1-independent regulation of *ZRT1*, *ZRT2,* and *ZRT3*. The expression of zinc homeostasis genes was also tested in the different media (SD without zinc) and similar results to LZM media were obtained (**Figure S7**).

How could Ssn6 act in zinc homeostasis? A complex of Ssn6 and Tup1 has been described as a transcriptional repressor in eukaryotes (Braun and Johnson 1997, Jimenez, Paroush et al. 1997, Grbavec, Lo et al. 1999, Mukai, Matsuo et al. 1999), which represses a range of genes including those involved in glucose repression, starch gradation, osmotic stress response, iron utilization, peptide and sterol uptake, hypoxia-repressed genes, DNA-damage inducible genes, and haploid- and α-specific genes (Keleher, Passmore et al. 1989, Balasubramanian, Lowry et al. 1993, Goutte and Johnson 1993, Treitel and Carlson 1995, Huang, Zhou et al. 1998, Park, Koh et al. 1999, Proft and Serrano 1999). In *C. albicans* Ssn6 was shown to regulate gene expression in complex with Tup1, independently of Tup1 (Hwang, Oh et al. 2003, Garcia-Sanchez, Mavor et al. 2005), and in combination with other DNA binding proteins (DBP) (Garcia-Sanchez, Mavor et al. 2005, Hernday, Lohse et al. 2016). The *CSR1* upregulation in *ssn6*Δ/Δ in the presence of zinc (in YPD) (**Figure 9 B**) suggests

that Ssn6 could function in complex with Csr1, other DBPs, or alone and repress the *CSR1* expression in the presence of zinc.

In *C. albicans* the expression of *ZRT1*, *ZRT2*, and *ZRT3* was not induced under zinc limitation in the *ssn6*Δ/Δ strain, although *CSR1* was transcribed. In addition to its repressor function, Ssn6 is known to activate the expression of certain genes, for example, the Ssn6-Sut1complex positively regulates hypoxic genes in *S. cerevisiae* (Regnacq, Alimardani et al. 2001). Recently, *C. albicans* Sut1, an ortholog of *S. cerevisiae* Sut1, was shown to positively control *CSR1* expression *in vivo* (Xu, Solis et al. 2015). Therefore, it seems likely that Ssn6 (in a complex with DBPs or Sut1) might activate the expression of *ZRT1, ZRT2,* and *ZRT3* genes under zinc limitation. Thus we propose Ssn6 to be a novel component in the regulation of *C. albicans* zinc homeostasis.

Conclusion

As both a commensal colonizer of the human gut and a pathogen that is able to disseminate through the blood to different organs, *C. albicans* can be exposed to host niches with dramatically different zinc content. Furthermore, the host regulates zinc availability during infections *via* both zinc overload and limitation. Therefore, *C. albicans* can only thrive within the host by controlling its zinc homeostasis.

We found that *C. albicans* cells exposed to limited zinc conditions activate oxidative stress resistance, zinc uptake, and mobilization of zinc storage, while transcription, translation, and biosynthesis processes are repressed. The growth defect of the *zrt2*Δ/Δ mutant under low zinc levels was expected, but we additionally found that the *zrt2*Δ/Δ mutant upregulates the zincophore and zinc storage mobilization systems even in the presence of zinc. We hypothesize Zrt2 to be a transceptor protein in *C. albicans*, possibly acting *via* Ste50, an adaptor protein involved in signal transduction cascades.

So far, two TFs are known to be zinc homeostasis regulators in *C. albicans*: Csr1 and Sut1 (Kim, Kil et al. 2008, Xu, Solis et al. 2015), while at least four TFs (Sfu1, Hap43, Sef1, and Rim101) are required for proper iron homeostasis (Baek, Li et al. 2008, Chen, Pande et al. 2011). In this work, we demonstrated that the *ssn6*Δ/Δ mutant fails to upregulate zinc uptake transporters, explaining its growth defect and we thus identified Ssn6 as a potential novel contributor to zinc-dependent gene regulation.

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Figure and Table legends

Figure 1. Transcriptional profiling of zinc homeostasis genes in the wild type under low zinc levels. *ZRT1* (**A**), *ZRT2* (**B**), *PRA1* (**C**), *ZRT3* (**D**), and *ZRC1* (**E**) transcript levels in the wild type were measured *via* microarray method. Asterisks indicate statistical significance compared to the 0 min time point (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

Figure 2. Top 10 most overrepresented GO terms in the wild type under zinc limitation conditions. GO terms enriched from upregulated (**A**) and downregulated (**B**) genes at both 120 and 240 min and from upregulated (**C**) and downregulated (**D**) genes at both 480 and 960 min.

Figure 3. Transcriptional profiling of genes differently expressed starting from 120 min until 1400 min in the wild type under low zinc levels. *SOD1* (**A**), *HSP70* (**B**), *EEP1* (**C**), and *ASN1* (**D**) transcript levels in the wild type were measured *via* microarray scanning. Asterisks indicate statistical significance compared to the 0 min time point (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

Figure 4. Transcriptional profiling of zinc homeostasis genes in the wild type and *zrt2*Δ/Δ. *PRA1*, *ZRT1*, and *ZRT3* transcript levels were measured in the wild type (**A1B1C1D1**) and *zrt2*Δ/Δ (**A2B2C2D2**) *via* microarray scanning at 4 and 16 h in LZM+5μM ZnSO4, at 24 h in LZM, and in 48h in LZM+300μM zinc citrate. Asterisks indicate statistical significance compared to the 0 min time point (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001; ns, p > 0.05).

Figure 5. Phenotypic profiling of the wild type and *zrt2*Δ/Δ. The wild type (**A**) and *zrt2*Δ/Δ (**B**) strains were pre-starved 24 h for zinc in LZM, and then grown in LZM, LZM+300μM zinc citrate, and LZM+300μM ZnSO4 with a starting OD of 0.005.

Figure 6. The relative growth of the wild type and mutant strains. The wild type and 149 mutant strains were grown in LZM for 24 h (starvation phase) (**A**), which was followed by growth in LZM for 48 h (limitation phase) (**B**). Afterwards, cells were transferred in LZM (2nd limitation phase) (**C**), in LZM+5μMZnSO4 (**D**) or in LZM+5μM zinc citrate (**E**) for 48 h (refeeding phases) (Figure S1). The relative growth was calculated as described in the materials and methods section and the complete list of values is preset in the Table S9.

Figure 7. Phenotypic profiling of the wild type, *ssn6*Δ/Δ, and *ssn6*Δ/Δ+*SSN6* under sufficient and low zinc levels. The wild type (**A**), *ssn6*Δ/Δ (**B**), and *ssn6*Δ/Δ+*SSN6* (**C**) strains were pre-starved 24 h for zinc, and then grown in LZM or in LZM+300μM ZnSO4 with a starting OD of 0.005.

Figure 8. Phenotypic profiling of wild type and *ssn6*Δ/Δ under sufficient and low iron levels. The wild type (**A**) and *ssn6*Δ/Δ (**B**) strains were pre-starved 24 h for iron and then grown in LM (limited media with 300μM ZnSO4 and 300μM FeCl3), in LIM+300μM ZnSO4, and in LZM+300μM FeCl3 with a starting OD of 0.005.

Figure 9. Transcriptional profiling of zinc homeostasis genes. *SSN6* (**A**), *CSR1* (**B**), *ZRT1* (**C**), *PRA1* (**D**), *ZRT2* (**E**), and *ZRT3* (**F**) transcript levels were quantified by qRT-PCR in wild type and all mutant strains after 4 h in YPD, LZM+300μM ZnSO4, and LZM. The expression was normalized to the transcript levels of *ACT1* in each strain. Asterisks indicate statistical significance compared to the wild type strain (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001); n.d. indicates not detected expression.

Table 1. The wild type zinc-responsive genes that are not regulated in *zrt2*Δ/Δ.

Table 2. The genes of the depleted mutants. The mutants that possess a growth defect (**A**) and a growth advantage (**B**) in comparison to the wild type.

Figures and Tables

Figure 1

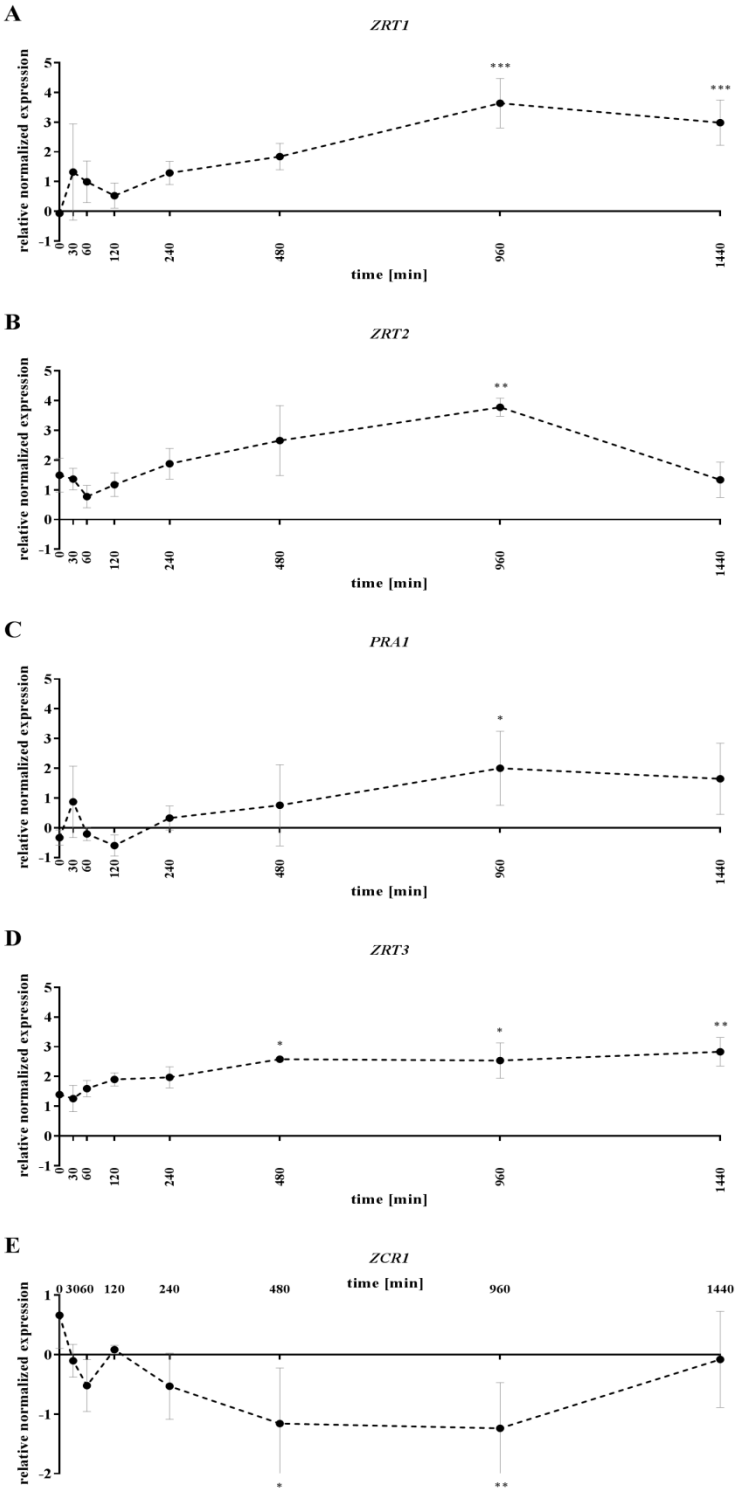
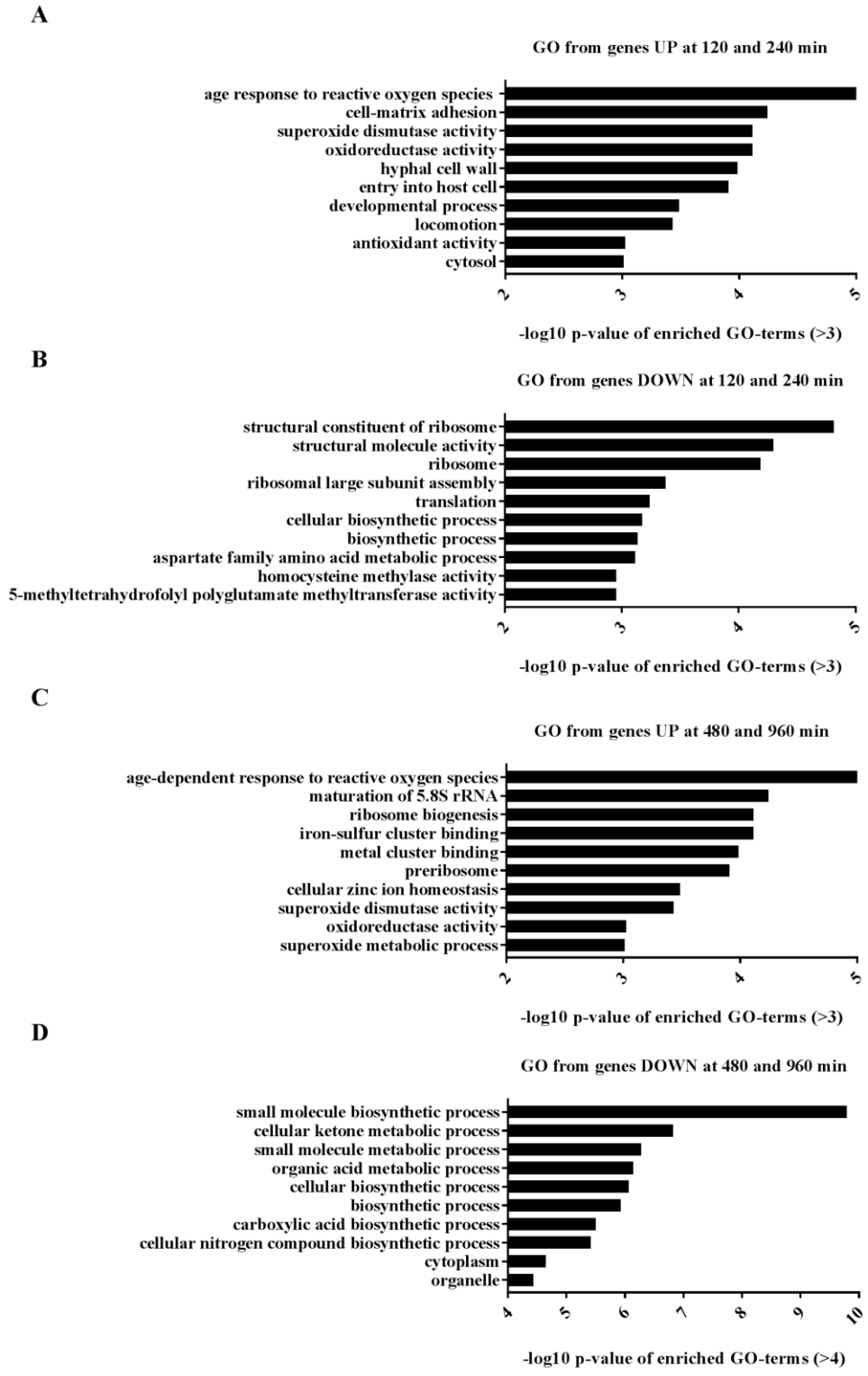


Figure 2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GO from genes UP at 120 and 240 min
age response to reactive oxygen species
cell-matrix adhesion
superoxide dismutase activity
oxidoreductase activity
hyphal cell wall
entry into host cell
developmental process
locomotion
antioxidant activity
cytosol
2
3
4
5
-log10 p-value of enriched GO-terms (>3)
B
GO from genes DOWN at 120 and 240 min
structural constituent of ribosome
structural molecule activity
ribosome
ribosomal large subunit assembly
translation
cellular biosynthetic process
biosynthetic process
aspartate family amino acid metabolic process
homocysteine methylase activity
5-methyltetrahydrofolyl polyglutamate methyltransferase activity
2
3
4
5
-log10 p-value of enriched GO-terms (>3)
C
GO from genes UP at 480 and 960 min
age-dependent response to reactive oxygen species
maturation of 5.8S rRNA
ribosome biogenesis
iron-sulfur cluster binding
metal cluster binding
preribosome
cellular zinc ion homeostasis
superoxide dismutase activity
oxidoreductase activity
superoxide metabolic process
2
3
4
5
-log10 p-value of enriched GO-terms (>3)
D
GO from genes DOWN at 480 and 960 min
small molecule biosynthetic process
cellular ketone metabolic process
small molecule metabolic process
organic acid metabolic process
cellular biosynthetic process
biosynthetic process
carboxylic acid biosynthetic process
cellular nitrogen compound biosynthetic process
cytoplasm
organelle
4
5
6
7
8
9
10
-log10 p-value of enriched GO-terms (>4)

Figure 3

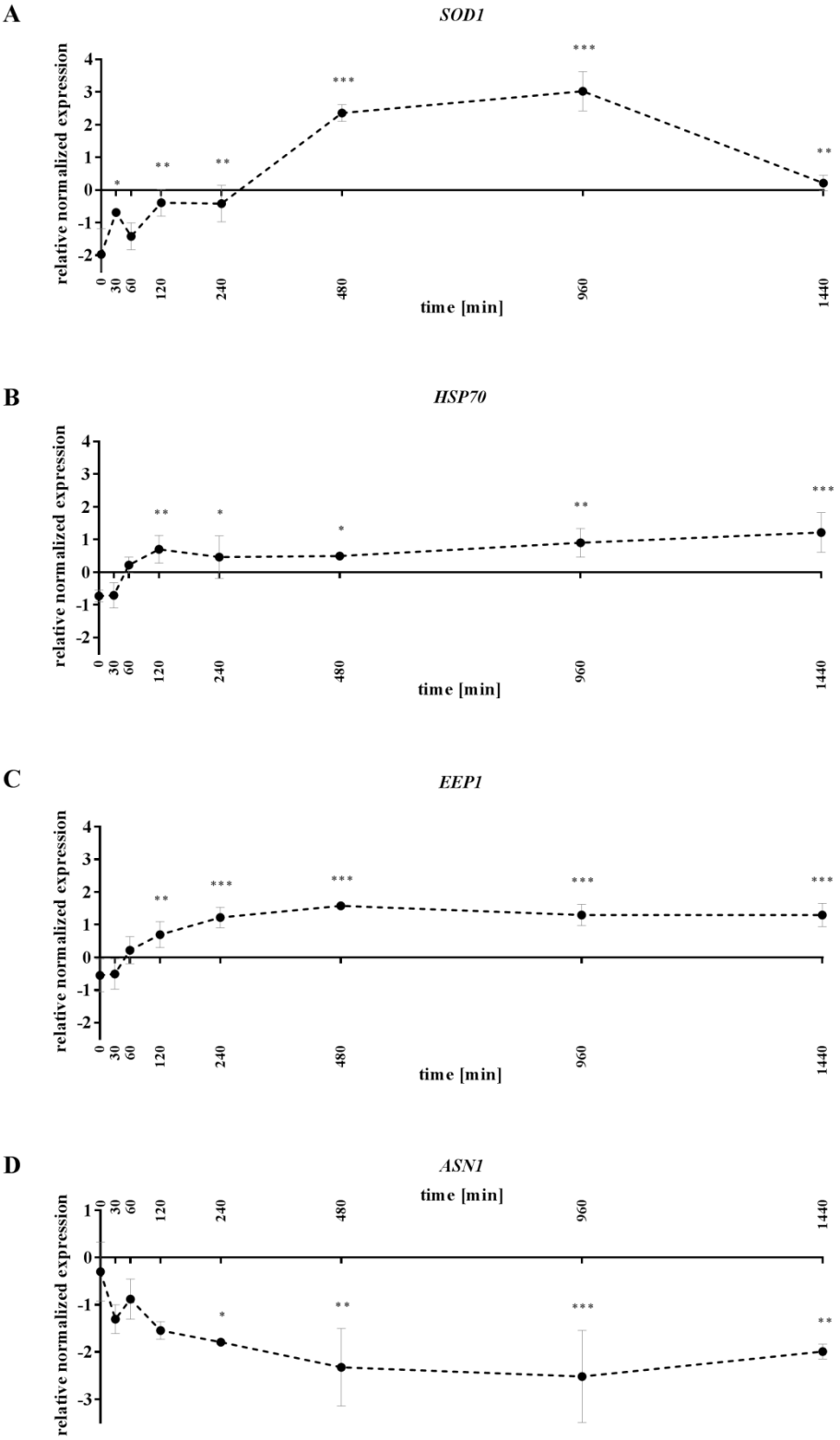
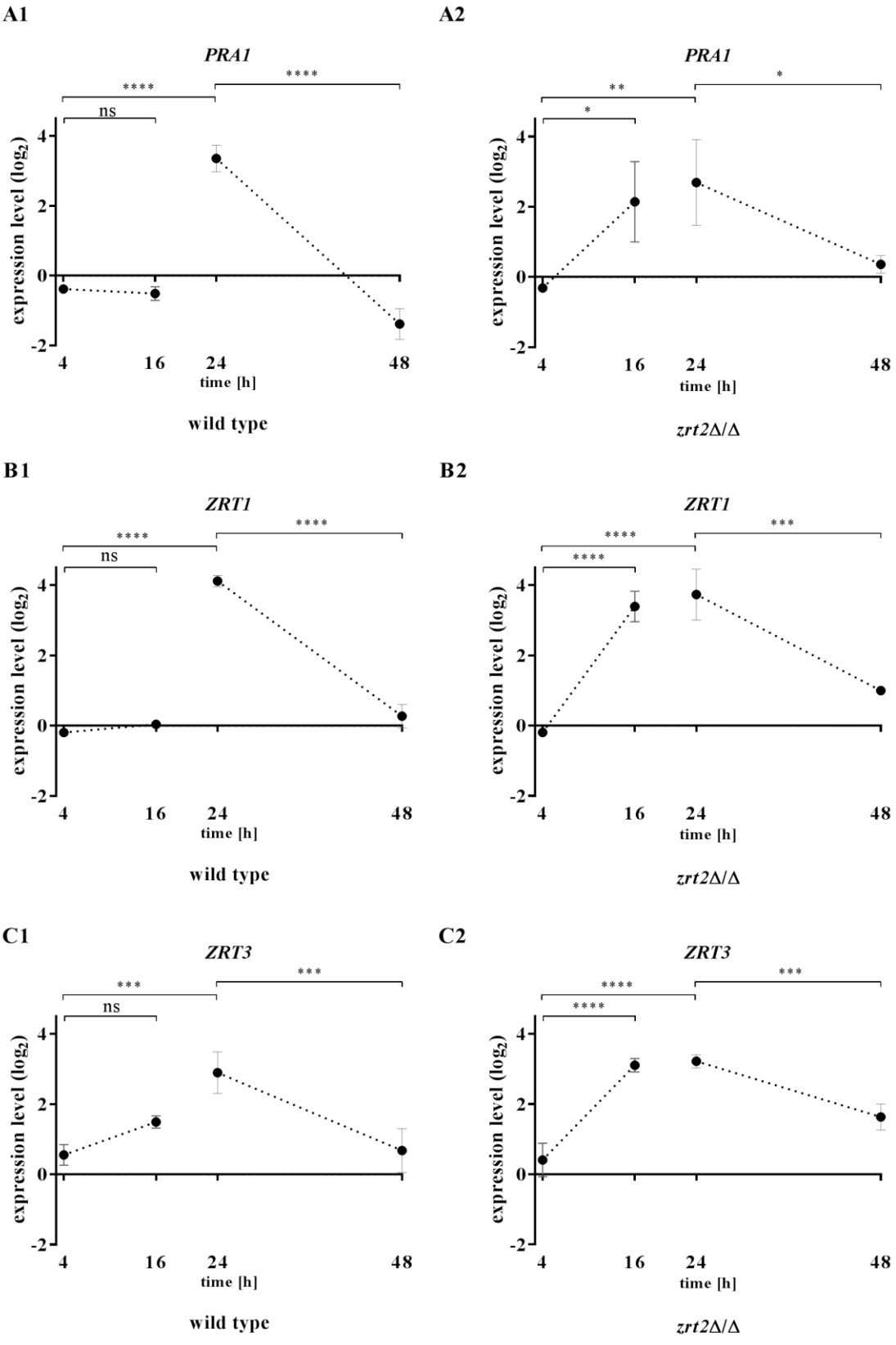

A
SOD1
relative normalized expression
time [min]
B
HSP70
relative normalized expression
time [min]
C
EEP1
relative normalized expression
time [min]
D
ASN1
relative normalized expression
time [min]

Figure 4


A1
PRA1
****
****
ns
expression level (log2)
4
2
0
-2
4
16
24
48
time [h]
wild type
A2
PRA1
**
*
*
expression level (log2)
time [h]
zrt2Δ/Δ
B1
ZRT1
****
****
ns
expression level (log2)
time [h]
wild type
B2
ZRT1
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expression level (log2)
time [h]
zrt2Δ/Δ
C1
ZRT3
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ns
expression level (log2)
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wild type
C2
ZRT3
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expression level (log2)
time [h]
zrt2Δ/Δ

Figure 5

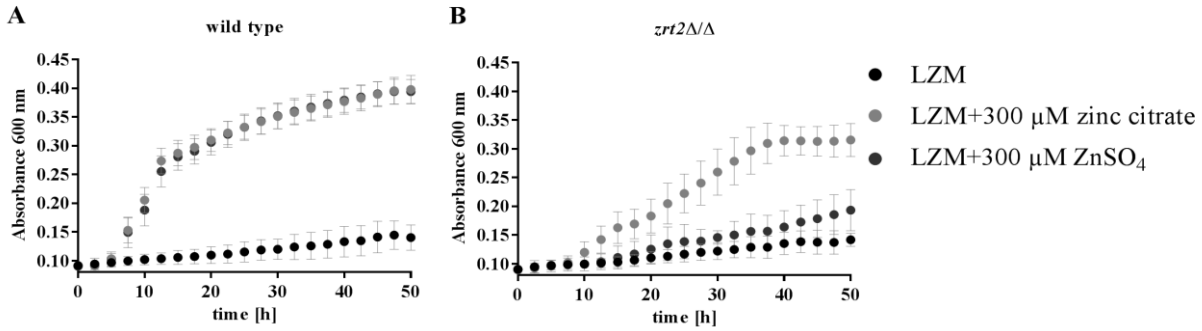


Figure 6

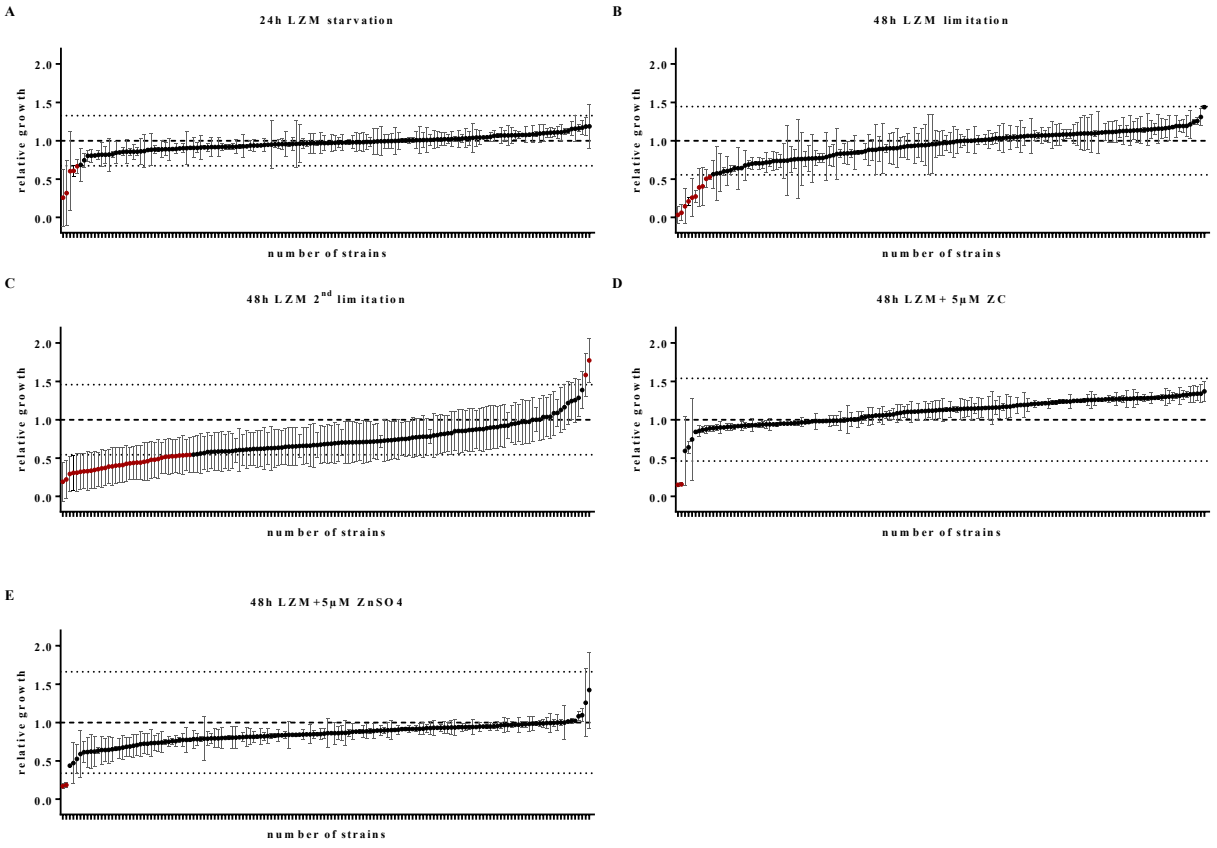


Figure 7

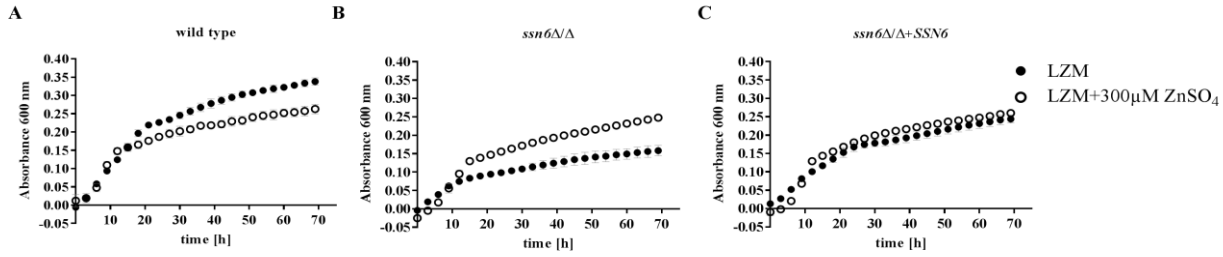


Figure 8

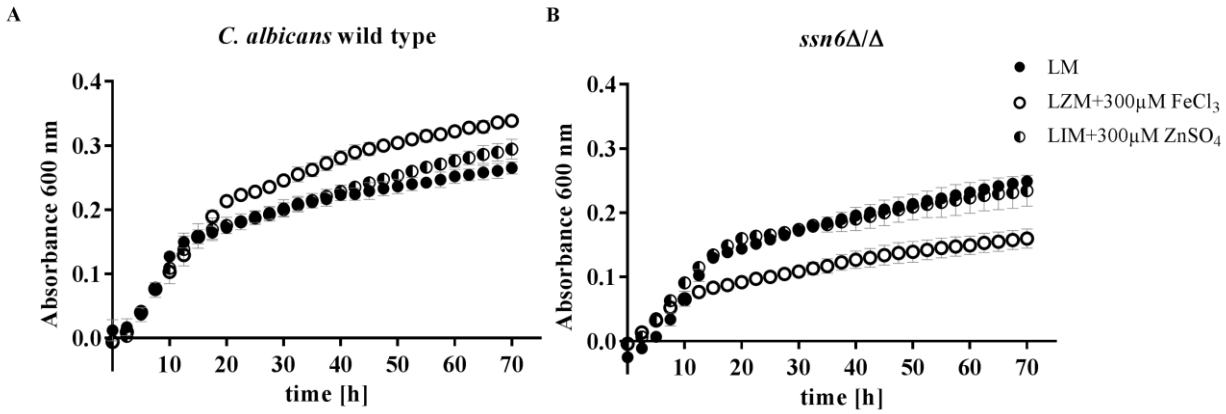


Figure 9

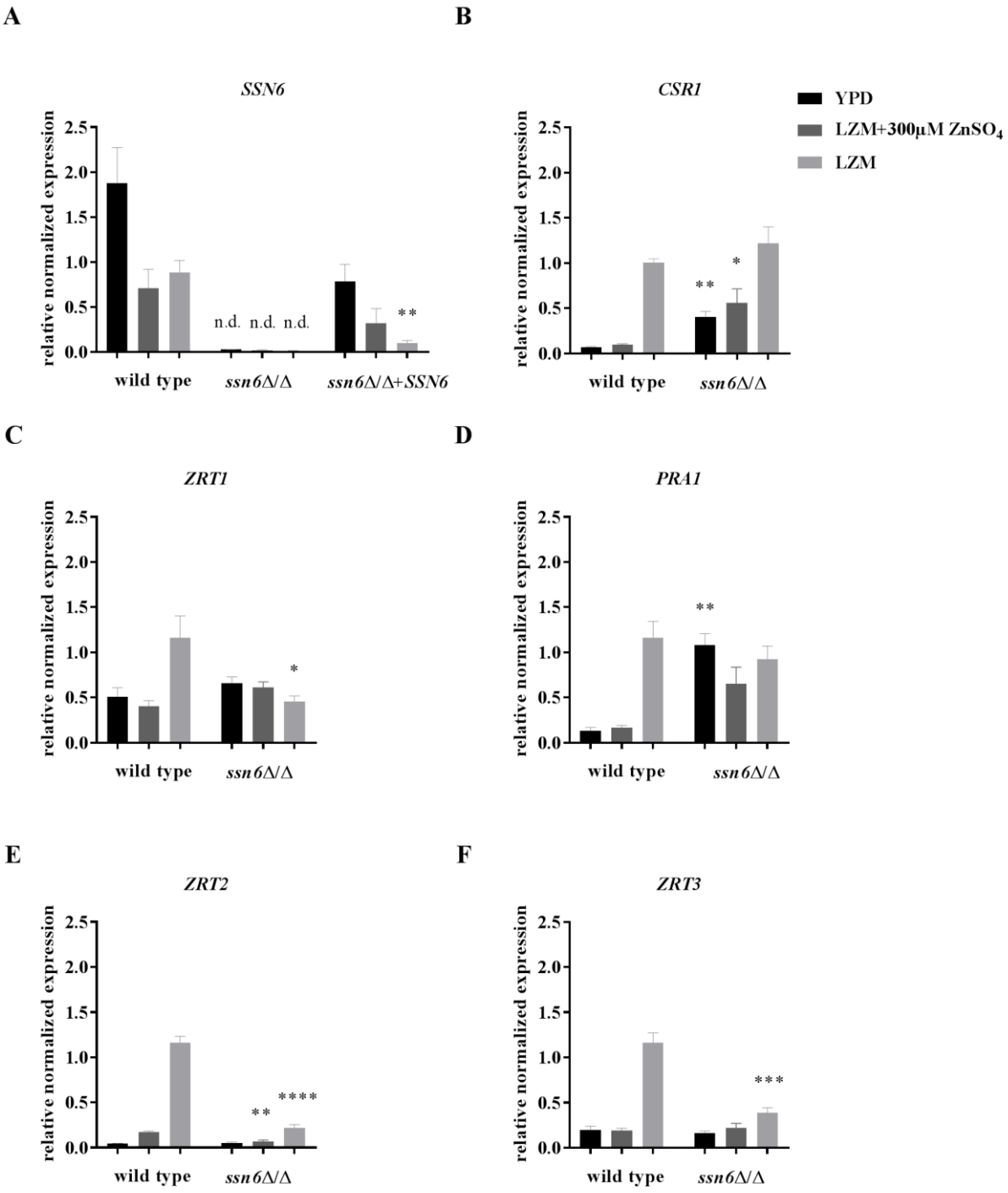

A
SSN6
relative normalized expression
2.5
2.0
1.5
1.0
0.5
0.0
n.d. n.d. n.d.
**
wild type
ssn6Δ/Δ
ssn6Δ/Δ+SSN6
B
CSR1
**
*
wild type
ssn6Δ/Δ
YPD
LZM+300µM ZnSO4
LZM
C
ZRT1
*
wild type
ssn6Δ/Δ
D
PRA1
**
wild type
ssn6Δ/Δ
E
ZRT2
**
****
wild type
ssn6Δ/Δ
F
ZRT3
***
wild type
ssn6Δ/Δ

Table 1

Name	Symbol	Description
Upregulated wild type genes and not regulated in *zrt2*Δ/Δ		
orf19.1795	*PUF3*	RNA-binding protein involved in regulation of mitochondrial biogenesis
orf19.2018		Protein with a predicted DnaJ chaperone domain and a CSL-type zinc finger; Spider biofilm induced
orf19.1642		Ortholog of S. cerevisiae Loc1, a nuclear protein involved in asymmetric localization of ASH1 mRNA in S. cerevisiae; Hap43-induced gene; Spider biofilm induced
orf19.1636	*STE50*	Protein with sterile alpha motif (SAM) and Ras-associated domain (RAD); similar to S. cerevisiae Rad50p, which is involved in signal transduction *via* interaction with and regulation of MAPKKK
orf19.1610		Putative protein of unknown function, transcription is activated in the presence of elevated CO2
orf19.1591	*ERG10*	Acetyl-CoA acetyltransferase; role in ergosterol biosynthesis; soluble in hyphae; changes in protein abundance associated with azole resistance; fluconazole or ketoconazole induced; macrophage-downregulated protein; GlcNAc-induced protein
orf19.2963		Has domain(s) with predicted NAD+ binding activity
orf19.2440	*RTT101*	Putative cullin subunit of E3 ubiquitin ligase complex, involved in response to DNA damage; induced by alpha pheromone in SpiderM medium
orf19.2049		Plasma membrane-associated protein; heterozygous null mutant displays sensitivity to virgineone; Spider biofilm induced
orf19.6722		Protein similar to S. cerevisiae Rad4p; downregulation associated with azole resistance
orf19.2619	*PHO113*	Putative constitutive acid phosphatase; Rim101-repressed; DTT-extractable; N-glycosylated; possibly an essential gene, disruptants not obtained by UAU1 method
Downregulated wild type genes and not regulated in *zrt2*Δ/Δ		
orf19.1863		Has domain(s) with predicted Rho guanyl-nucleotide exchange factor activity, role in regulation of Rho protein signal transduction and intracellular localization
orf19.4631	*ERG251*	C-4 sterol methyl oxidase; role in ergosterol biosynthesis; Hap43-induced; ketoconazole-induced; amphotericin B, caspofungin repressed; possibly essential gene, disruptants not obtained by UAU1 method; Spider biofilm repressed
orf19.2772	*HOS3*	Histone deacetylase; similar to S. cerevisiae Hos3p; greater expression and longer mRNA in white cells, compared to opaque cells; has conserved deacetylation motif
orf19.5069		Ortholog of S. cerevisiae Sae3; meiosis specific protein involved in DMC1-dependent meiotic recombination in S. cerevisiae; Spider biofilm induced

Table 2

A

Genes of depleted mutants									
24 h LZM		**48 h LZM**		**48 h 2nd LZM**		**48 h LZM+5µM ZS**		**48 h LZM+5µM ZC**	
orf19.5558	*RBF1*	orf19.5558	*RBF1*	orf19.5558	*RBF1*	orf19.5558	*RBF1*	orf19.5558	*RBF1*
orf19.7150	*NRG1*	orf19.7150	*NRG1*	orf19.7150	*NRG1*	orf19.7150	*NRG1*	orf19.7150	*NRG1*
orf19.6798	*SSN6*	orf19.6798	*SSN6*	orf19.6798	*SSN6*				
orf19.6124	*ACE2*	orf19.3794	*CSR1*	orf19.3794	*CSR1*				
orf19.4545	*SWI4*	orf19.3063	*HFL1*	orf19.3063	*HFL1*				
		orf19.2808	*ZCF16*	orf19.2808	*ZCF16*				
		orf19.2842	*GZF3*	orf19.2842	*GZF3*				
		orf19.2745	*UME7*	orf19.2745	*UME7*				
		orf19.3625		orf19.3625					
		orf19.3127	*CZF1*	orf19.3127	*CZF1*				
				orf19.2646	*ZCF13*				
				orf19.5251	*ZCF30*				
				orf19.454	*SFL1*				
				orf19.6781	*ZFU2*				
				orf19.5910					
				orf19.5729	*FGR17*				
				orf19.4941	*TYE7*				
				orf19.5133	*ZCF29*				
				orf19.2753	*ZCF15*				
				orf19.3190	*HAL9*				
				orf19.921	*HMS1*				
				orf19.4318	*MIG1*				
				orf19.1499	*CTF1*				
				orf19.3193	*FCR3*				
				orf19.6109	*TUP1*				

orf19.2961	
orf19.3188	*TAC1*
orf19.4752	*MSN4*
orf19.4647	*HAP3*
orf19.7401	*ISW2*
orf19.1069	*RPN4*
orf19.2647	*ZCF14*
orf19.6680	*FGR27*
orf19.3305	*ZCF17*
orf19.4225	*LEU3*
orf19.2730	
orf19.1253	*PHO4*

B

Genes of depleted mutants	
48 h 2nd LZM	
orf19.1718	*ZCF8*
orf19.1685	*ZCF7*

Supplementary Figure and Table legends

Figure S1. A scheme of the workflow for the transcription factor deletion library screening.

Figure S2. Hierarchical clustering of the wild type transcriptional response to zinc limitation.

Figure S3. Overall transcriptional response to zinc limitation in the wild type. 11 genes were significantly upregulated at both 120 and 240 min (**A**). 8 genes were significantly downregulated at both 120 and 240 min (**B**). 38 genes were significantly upregulated at both 480 and 960 min (**C**). 57 genes were significantly downregulated at both 480 and 960 min (**D**).

Figure S4. Overall transcriptional response to zinc limitation in wild type and *zrt2*Δ/Δ. 97 genes are significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate in the wild type (**A1**). 81 significantly upregulated genes only in LZM and not in LZM+5μM $ZnSO_4$ in the wild type (**A2**). 78 genes were significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate in *zrt2*Δ/Δ (**B**). 15 genes were significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate in the wild type (**C1**). 13 significantly downregulated genes only in LZM and not in LZM+5μM $ZnSO_4$ in the wild type (**C2**). 107 genes were significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate in *zrt2*Δ/Δ (**D**).The significantly regulated genes for *zrt2*Δ/Δ in LZM were not dissected, as the mutant senses zinc limitation in LZM+5μM $ZnSO_4$.

Figure S5. Top 10 most over-represented GO terms from zinc-responsive genes in wild type and *zrt2*Δ/Δ. GO terms from significantly upregulated genes in wild type (**A**) and in *zrt2*Δ/Δ (**B**). GO terms from significantly downregulated genes in wild type (**C**) and in *zrt2*Δ/Δ (**D**).

Figure S6. Overall transcriptional response of common and specific zinc response genes in the wild type and *zrt2*Δ/Δ strains. 67 genes were significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate in the wild type, but not in *zrt2*Δ/Δ (**A**). 11 genes were significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate in the wild type, but not in *zrt2*Δ/Δ (**B**).

Figure S7. Transcriptional profiling of zinc homeostasis genes. C*SR1* (**A**), *PRA1* (**B**), *ZRT1* (**C**), *ZRT2* (**D**), and *ZRT3* (**E**) transcript levels were measured by qRT-PCR in wild type and all mutant strains after 4 h in SD without zinc. The expression was normalized to the transcript levels of *ACT1* in each strain. Asterisks indicate statistical significance compared to the wild type strain (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Table S1. The list of *C. albicans* strains used in this study.

Table S2. The list of primers used in this study.

Table S3. LZM and LIM medium composition.

Table S4. The complete list of GO terms obtained from upregulated (**A**) and downregulated (**B**) genes at both 120 and 240 min.

Table S5. The complete list of GO terms obtained from upregulated (**A**) and downregulated (**B**) genes at both 480 and 960 min.

Table S6. The complete list of GO terms obtained from the wild type zinc-responsive genes. GO terms from genes significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate (**A**) and from genes significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate in the wild type (**B**).

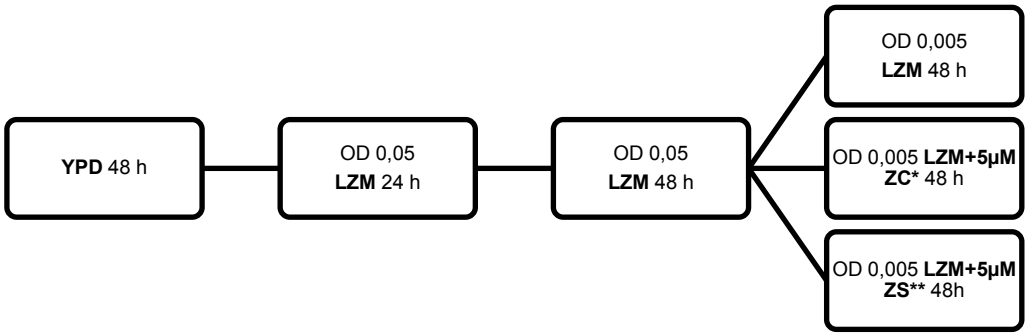
Table S7. The complete list of GO terms obtained from *zrt2*Δ/Δ zinc-responsive genes. GO terms from genes significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate (**A**) and from genes significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate in *zrt2*Δ/Δ (**B**).

Table S8. The list of the wild type zinc-responsive genes that are not regulated in *zrt2*Δ/Δ. The wild type genes significantly upregulated in LZM and significantly downregulated in LZM+300μM zinc citrate (**A**). The wild type genes significantly downregulated in LZM and significantly upregulated in LZM+300μM zinc citrate (**B**).

Table S9. The relative growth values of the wild type and mutant strains.

Supplementary Figures and Tables

Figure S1



* ZS - $ZnSO_4$

** ZC - zinc citrate

Figure S2

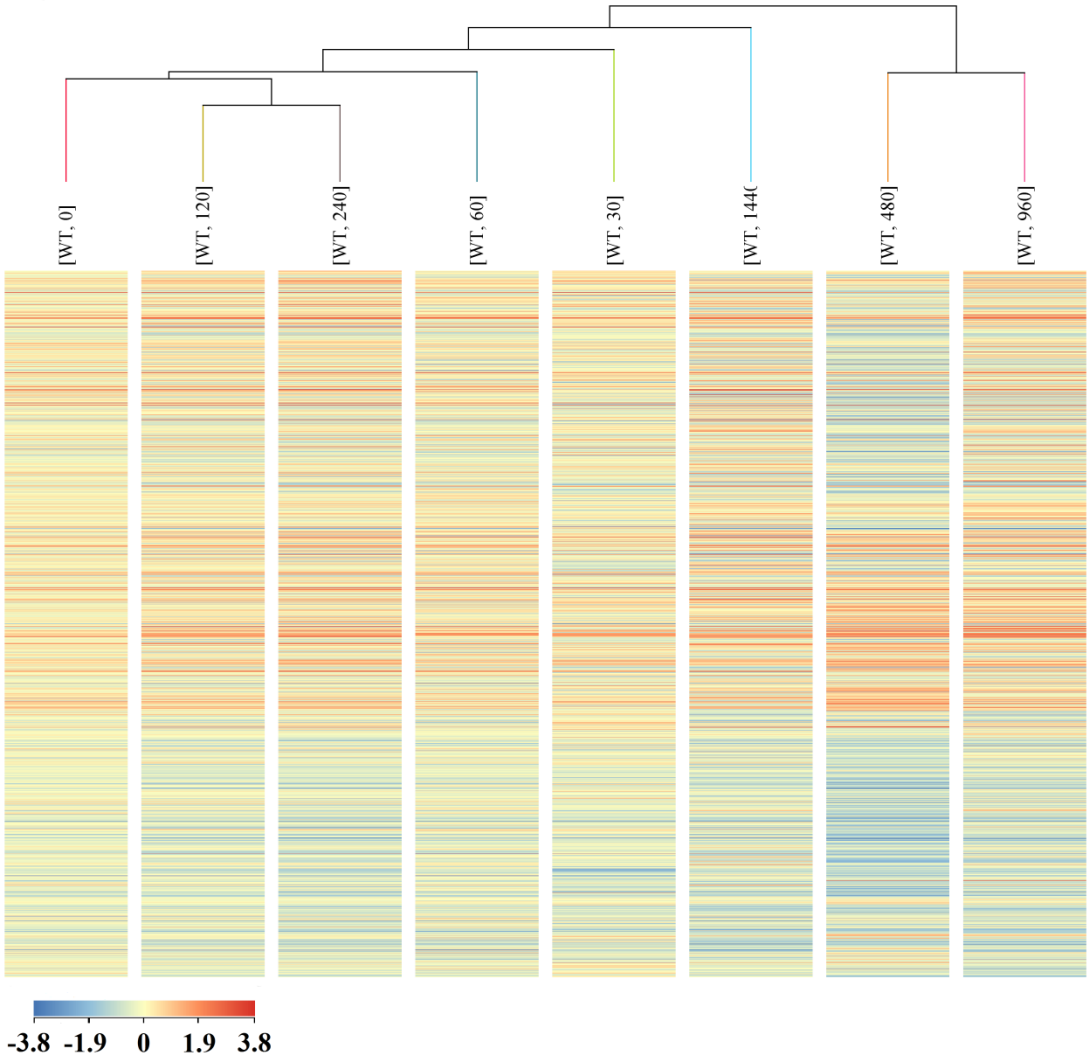


Figure S3

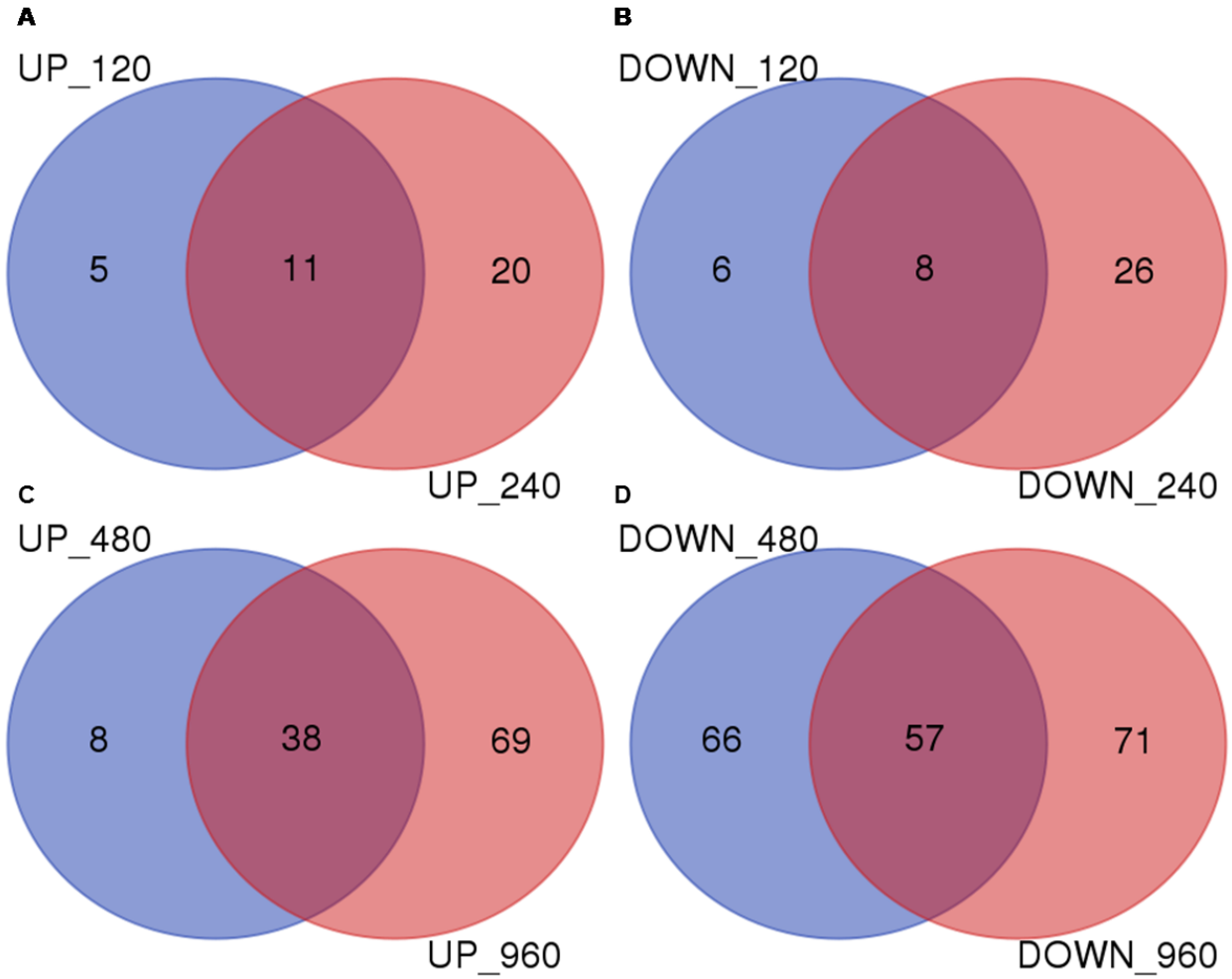

A
UP_120
5
11
20
UP_240
B
DOWN_120
6
8
26
DOWN_240
C
UP_480
8
38
69
UP_960
D
DOWN_480
66
57
71
DOWN_960

Figure S4

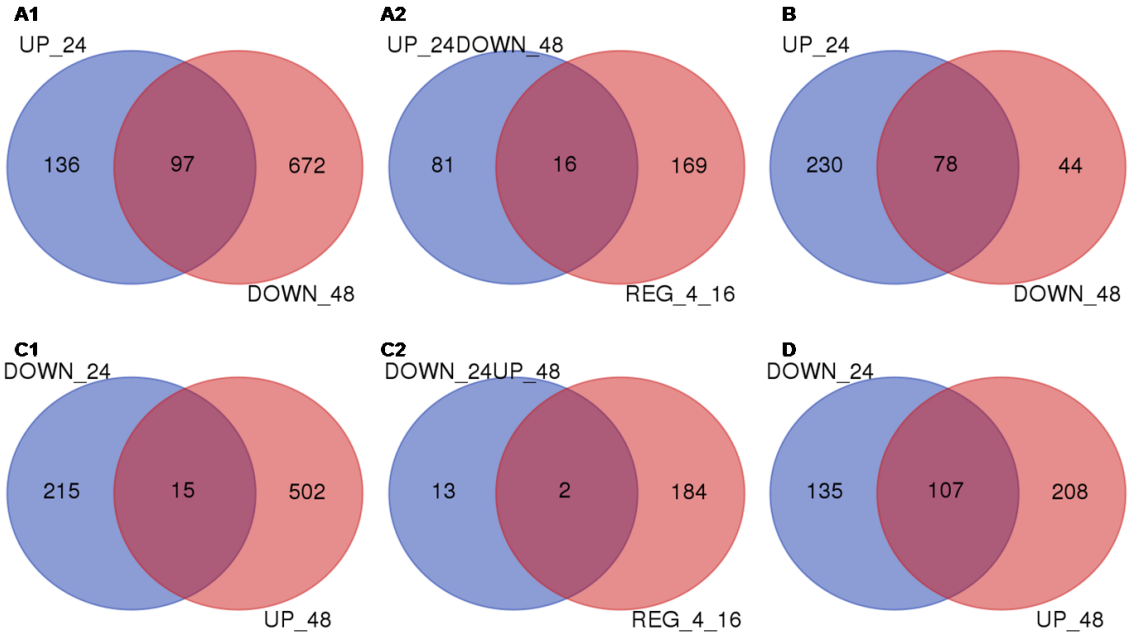

A1
UP_24
136
97
672
DOWN_48
A2
UP_24DOWN_48
81
16
169
REG_4_16
B
UP_24
230
78
44
DOWN_48
C1
DOWN_24
215
15
502
UP_48
C2
DOWN_24UP_48
13
2
184
REG_4_16
D
DOWN_24
135
107
208
UP_48

Figure S5

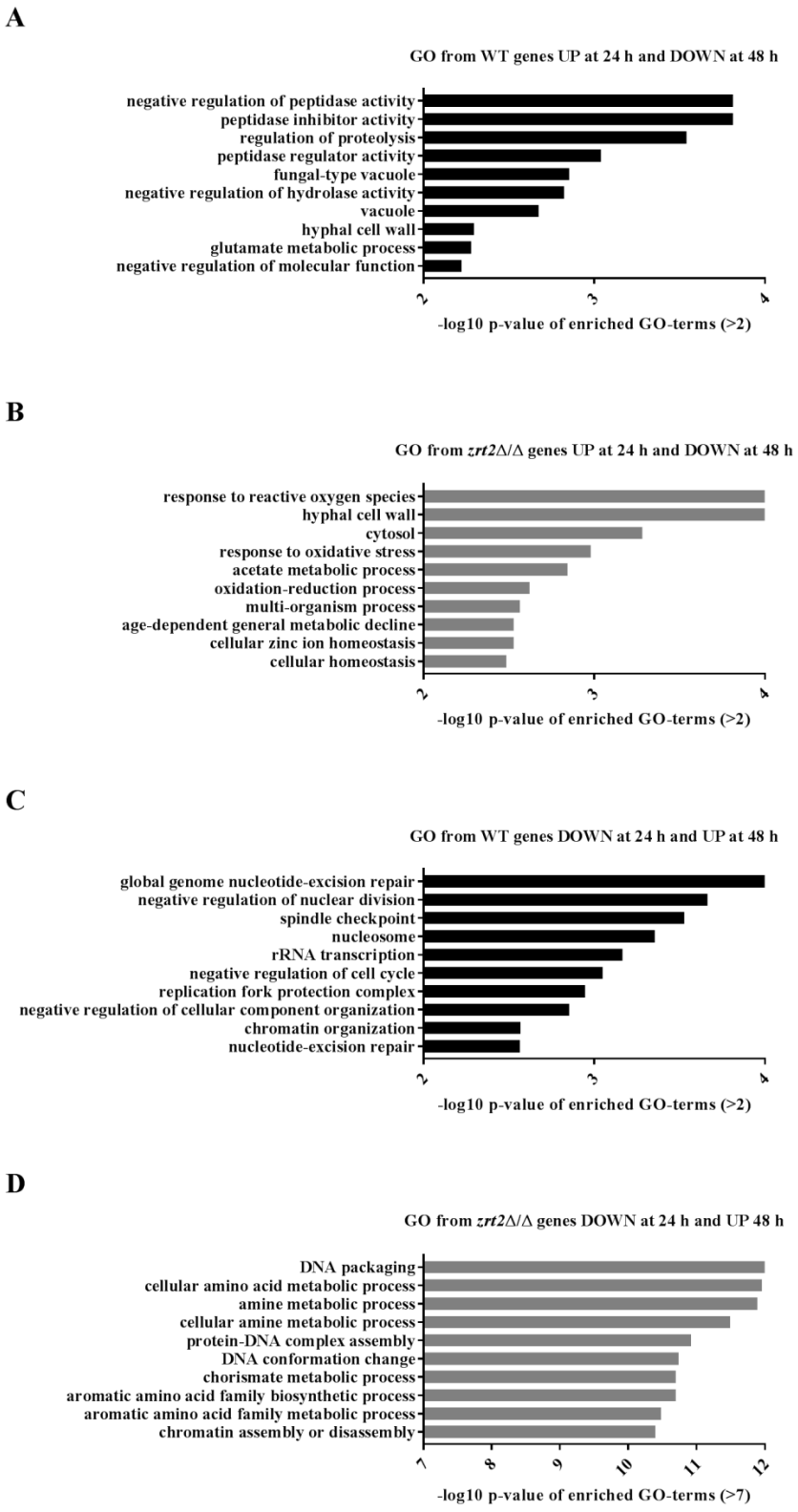

A
GO from WT genes UP at 24 h and DOWN at 48 h
negative regulation of peptidase activity
peptidase inhibitor activity
regulation of proteolysis
peptidase regulator activity
fungal-type vacuole
negative regulation of hydrolase activity
vacuole
hyphal cell wall
glutamate metabolic process
negative regulation of molecular function
2
3
4
-log10 p-value of enriched GO-terms (>2)
B
GO from zrt2Δ/Δ genes UP at 24 h and DOWN at 48 h
response to reactive oxygen species
hyphal cell wall
cytosol
response to oxidative stress
acetate metabolic process
oxidation-reduction process
multi-organism process
age-dependent general metabolic decline
cellular zinc ion homeostasis
cellular homeostasis
2
3
4
-log10 p-value of enriched GO-terms (>2)
C
GO from WT genes DOWN at 24 h and UP at 48 h
global genome nucleotide-excision repair
negative regulation of nuclear division
spindle checkpoint
nucleosome
rRNA transcription
negative regulation of cell cycle
replication fork protection complex
negative regulation of cellular component organization
chromatin organization
nucleotide-excision repair
2
3
4
-log10 p-value of enriched GO-terms (>2)
D
GO from zrt2Δ/Δ genes DOWN at 24 h and UP 48 h
DNA packaging
cellular amino acid metabolic process
amine metabolic process
cellular amine metabolic process
protein-DNA complex assembly
DNA conformation change
chorismate metabolic process
aromatic amino acid family biosynthetic process
aromatic amino acid family metabolic process
chromatin assembly or disassembly
7
8
9
10
11
12
-log10 p-value of enriched GO-terms (>7)

Figure S6

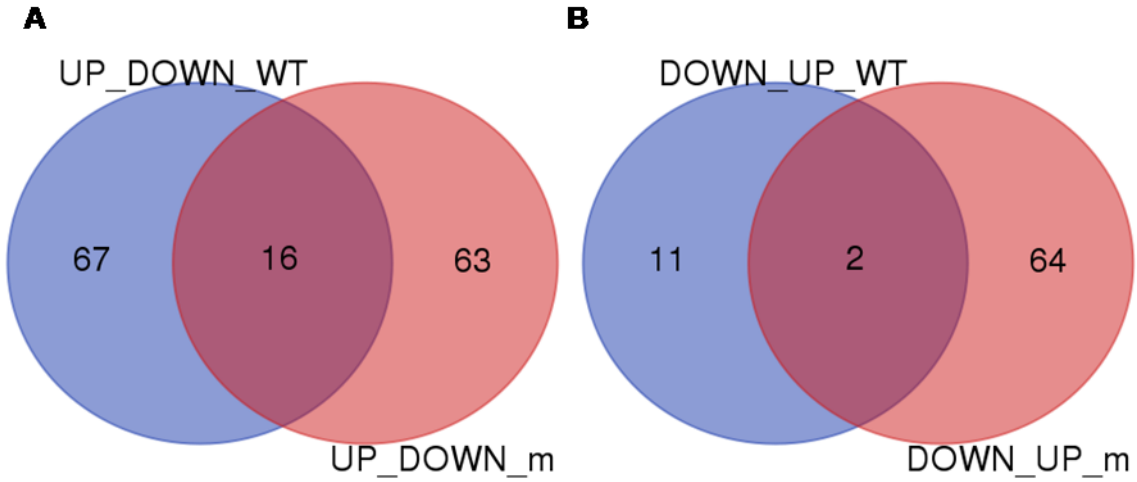


Figure S7

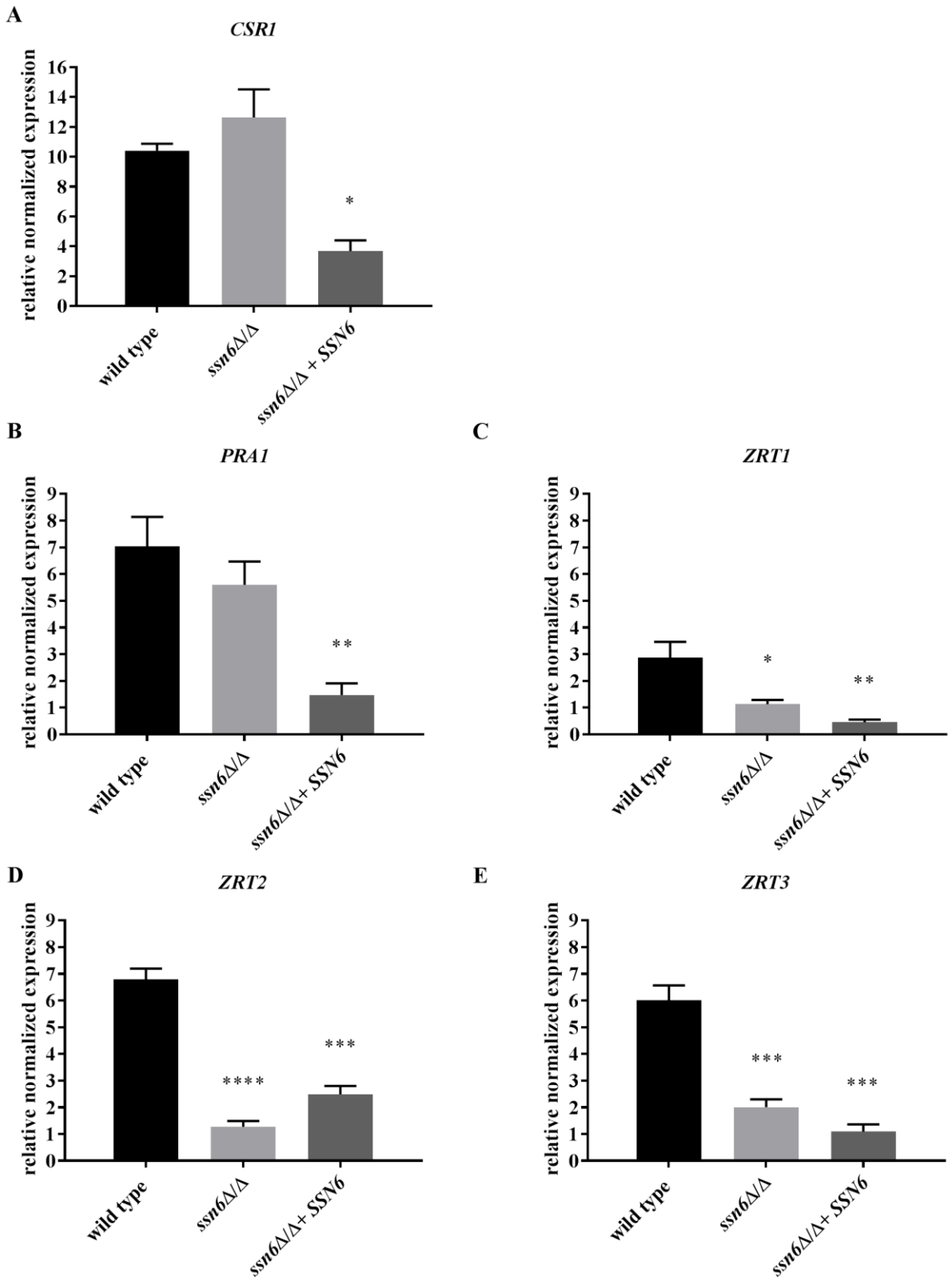

A
CSR1
relative normalized expression
wild type
ssn6Δ/Δ
ssn6Δ/Δ + SSN6
B
PRA1
C
ZRT1
D
ZRT2
E
ZRT3

Table S1

Strain name	Genotype	Reference	Unique identifier (Hubelab)
BWP17	ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG	(Wilson, Davis et al. 1999)	M130
BWP17+CIp30 isogenic "wild type"	ura3::λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG +CIp30	(Mayer, Wilson et al. 2012)	M1477
*zrt2*Δ/ Δ	*zrt2*::*HIS1*/*zrt2*::*ARG4* +Cip10	(Crawford, Lehtovirta-Morley et al. 2018)	M2163
SN152 wild type (Homann)	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ *URA3*/ura3Δ::imm 434 *IRO1*/iro1Δ::imm 434	(Homann, Dea et al. 2009)	M2343
ssn6 Δ/ Δ	*ssn6*::*HIS1*/*ssn6*::*ARG4* +Cip10	This study	M2463
ssn6 Δ/ Δ+*SSN6*	*ssn6*::*HIS1*/*ssn6*::*ARG4* +Cip10-*SSN6*	This study	M2464

Table S2

Name	Sequence 5->3'	Purpose
act1-f	tcagaccagctgatttaggtttg	qRT-PCR
act1-r	gtgaacaatggatggaccag	
rtfssn6	aaaacccgtggagtcacaac	
rtrssn6	attggattctggtgcctttg	
rtf60csr1	gtggagcatcatttgcagtg	
rtr60csr1	tattacacccgggccattta	
rtf59zrt1	acgctgtgacttgtgaaagagt	
rtr59zrt1	cacattaattgcgcatgagtc	
rtf58,4zrt2	cagacacagatatactccatggatt	
rtr58,4zrt2	aattcgtgtgccatcaattc	
fprtzrt3 61.2	caacaaccatagtgggca	
rprtzrt3 61.0	cttaaacaaattaatatccccatga	
rtpra1 59.54 fpr	tgaggtcgttggtcattttg	
rtpra1 59.41 rpr	accggagcatagttgggata	
ef1b-f	agtcattgaacgaattcttggctg	gDNA contamination

ef1b-r	tcttcatcaacttcatcatcagaacc	
ura-f2 rpf-1	ggagttggattagatgataaaggtgatgg gagcagtgtacacacacacatcttg	conrormation of plasmid integration
delcafpssn6 delcarpssn6	gtatttctgttttgatatacatttttgaaacaaaacaggtcaaaaccccctttttttggtagctgttacg actaatttttttttcattaacaaagatccggaaaggaagcttcgtacgctgcaggtc aaatgttaacaacaattgtatacatccccccccccatcccctaccccgtcaaatatatatatatattcaa ctatcatatacaatttcaccactttttctttatcttctgatatcatcgatgaattcgag	mutant generation
beffssn6 afrssn6	ccatttccatttccatttcc ctggtgatgataacgatgatga	colohy PCR
intrssn6	gctttgcagcttgttgatgt	colony PCR
befssn6_ov aftssn6_ov	tcgataccgtcgacccttctctgccccataaaattca gggaacaaaagctggcaccaccaccaccataaatg	Clp10-*SSN6*
rpf-f1	gagcagtgtacacacacacatcttg	(Mayer, Wilson et al. 2012)
ura3-f2	ggagttggattagatgataaaggtgatgg	(Gola, Martin et al. 2003)
his1-f2	ggacgaattgaagaaagctggtgcaaccg	(Gola, Martin et al. 2003)
his1-r2	caacgaaatggcctcccctaccacag	(Gola, Martin et al. 2003)
arg4-f2	ggatatgttggctactgatttagc	(Gola, Martin et al. 2003)
arg4-r2	aatggatcagtggcaccggtg	(Gola, Martin et al. 2003)

Table S3

EDTA (1) and sodium citrate (7) stocks were first adjusted to pH 8 and pH 4.2 respectively.

Stock	Fold conc.	Component	Stock conc. (M)	Final conc. (M)	Vol.
1	500	$Na_2EDTA.2H_2O$	$5.0x10^{-1}$	$1.0x10^{-3}$	1mL
2	100	$MgSO_4.7H_2O$ NaCl	$5.0x10^{-1}$ $1.0x10^{-1}$	$5.0x10^{-3}$ $1.0x10^{-3}$	5mL
3	100	$CaCl_2.2H_2O$	$1.0x10^{-1}$	$1.0x10^{-3}$	5mL
4	100	Uridine L-Histidine L-Leucine L-Lysine	$4.0x10^{-2}$ $5.0x10^{-2}$ $7.6x10^{-2}$ $7.0x10^{-2}$	$4.0x10^{-4}$ $5.0x10^{-4}$ $7.6x10^{-4}$ $7.0x10^{-4}$	5mL

5	100	$(NH_4)_2SO_4$	3.8	$3.8x10^{-2}$	5mL
6	100	KH_2PO_4	$1.0x10^{-1}$	$1.0x10^{-3}$	5mL
7	50	$Na_3Citrate.2H_2O$	1.0	$2.0x10^{-2}$	10mL
8	20	D-glucose	$2.2x10^{-1}$	$1.1x10^{-2}$	25mL
9	1000	d-Biotin Ca Pantothenate myo-Inositol Pyridoxin Thiamin.HCl	$1.6x10^{-5}$ $1.7x10^{-3}$ $1.0x10^{-2}$ $2.0x10^{-3}$ $1.0x10^{-3}$	$1.6x10^{-8}$ $1.7x10^{-6}$ $1.0x10^{-5}$ $2.0x10^{-6}$ $1.0x10^{-6}$	0.5mL
10	10000	H_3BO_3 $CuSO_4.5H_2O$ KI $MnCl_2.4H_2O$ $Na_2MoO_4.2H_2O$ $FeCl_3$	$1.0x10^{-1}$ $2.0X10^{-3}$ $5.0x10^{-3}$ $2.5X10^{-1}$ $1.0x10^{-2}$ $5.0X10^{-1}$	$1.0x10^{-5}$ $2.0X10^{-7}$ $5.0x10^{-7}$ $2.5X10^{-5}$ $1.0x10^{-6}$ $1.0X10^{-6}$*	50µl

* Prepared medium was supplemented with 50µM $FeCl_3$

Table S4

A

Term ID	Description	log_{10} p
GO:0001320	age-dependent response to reactive oxygen species involved in chronological cell aging	-5.5601
GO:0007160	cell-matrix adhesion	-4.2400
GO:0004784	superoxide dismutase activity	-4.1154
GO:0016721	oxidoreductase activity, acting on superoxide radicals as acceptor	-4.1154
GO:0030446	hyphal cell wall	-3.9856
GO:0030260	entry into host cell	-3.9102
GO:0032502	developmental process	-3.4867
GO:0040011	locomotion	-3.4328
GO:0016209	antioxidant activity	-3.0251
GO:0005829	cytosol	-3.0126
GO:0030312	external encapsulating structure	-2.9753
GO:0051704	multi-organism process	-2.5448
GO:0006801	superoxide metabolic process	-2.4587
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	-2.2680
GO:0072593	reactive oxygen species metabolic process	-2.1584
GO:0006616	SRP-dependent cotranslational protein targeting to membrane, translocation	-2.1584
GO:0018456	aryl-alcohol dehydrogenase (NAD+) activity	-2.1584
GO:0055114	oxidation-reduction process	-2.0618
GO:0006882	cellular zinc ion homeostasis	-2.0618
GO:0006817	phosphate ion transport	-1.9830
GO:0006979	response to oxidative stress	-1.9572
GO:0031349	positive regulation of defense response	-1.9399

GO:0051090	regulation of sequence-specific DNA binding transcription factor activity	-1.9164
GO:0005315	inorganic phosphate transmembrane transporter activity	-1.8079
GO:0015114	phosphate ion transmembrane transporter activity	-1.7625
GO:0010035	response to inorganic substance	-1.7243
GO:0010226	response to lithium ion	-1.6840
GO:0042221	response to chemical	-1.6177
GO:0034605	cellular response to heat	-1.5604
GO:0007568	aging	-1.5561
GO:0035821	modification of morphology or physiology of other organism	-1.5344
GO:0009636	response to toxic substance	-1.5344
GO:0015103	inorganic anion transmembrane transporter activity	-1.5344
GO:0016491	oxidoreductase activity	-1.5058
GO:0007571	age-dependent general metabolic decline	-1.4627
GO:0015698	inorganic anion transport	-1.4440
GO:0033554	cellular response to stress	-1.3904
GO:0048584	positive regulation of response to stimulus	-1.3697
GO:0006950	response to stress	-1.3530
GO:0042277	peptide binding	-1.3530
GO:0000302	response to reactive oxygen species	-1.3508
GO:0009986	cell surface	-1.3390
GO:0070887	cellular response to chemical stimulus	-1.3369
GO:0072507	divalent inorganic cation homeostasis	-1.3050
GO:0071285	cellular response to lithium ion	-1.2783

B

Term ID	Description	$\log_{10}$ p
GO:0003735	structural constituent of ribosome	-4.8107
GO:0005198	structural molecule activity	-4.2985
GO:0005840	ribosome	-4.1882
GO:0000027	ribosomal large subunit assembly	-3.3759
GO:0006412	translation	-3.2383
GO:0044249	cellular biosynthetic process	-3.1740
GO:0009058	biosynthetic process	-3.1349
GO:0009066	aspartate family amino acid metabolic process	-3.1169
GO:0003871	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (homocysteine methylase)activity	-2.9557
GO:0042085	5-methyltetrahydropteroyltri-L-glutamate (5-methyltetrahydrofolyl polyglutamate)-dependent methyltransferase activity	-2.9557
GO:0070925	organelle assembly	-2.7016
GO:0006529	asparagine biosynthetic process	-2.6549
GO:0004066	asparagine synthase (glutamine-hydrolyzing) activity	-2.6549
GO:0042273	ribosomal large subunit biogenesis	-2.6358
GO:0071826	ribonucleoprotein complex subunit organization	-2.5181
GO:0008097	5S rRNA binding	-2.4790
GO:0008172	S-methyltransferase activity	-2.4790
GO:0004523	RNA-DNA hybrid ribonuclease activity	-2.3542
GO:0005625	(obsolete) soluble fraction	-2.3498
GO:0043228	non-membrane-bounded organelle	-2.3100

GO:0043232	intracellular non-membrane-bounded organelle	-2.3100
GO:0009309	amine biosynthetic process	-2.1308
GO:0008152	metabolic process	-1.9643
GO:0016053	organic acid biosynthetic process	-1.9206
GO:0034645	cellular macromolecule biosynthetic process	-1.8467
GO:0009059	macromolecule biosynthetic process	-1.8405
GO:0005737	cytoplasm	-1.8121
GO:0009986	cell surface	-1.7209
GO:0044267	cellular protein metabolic process	-1.6557
GO:0010467	gene expression	-1.6490
GO:0031349	positive regulation of defense response	-1.6376
GO:0022625	cytosolic large ribosomal subunit	-1.6176
GO:0000267	(obsolete) cell fraction	-1.6067
GO:0019843	rRNA binding	-1.5985
GO:0034622	cellular macromolecular complex assembly	-1.5824
GO:0019538	protein metabolic process	-1.5716
GO:0044238	primary metabolic process	-1.5600
GO:0035821	modification of morphology or physiology of other organism	-1.5459
GO:0051817	modification of morphology or physiology of other organism involved in symbiotic interaction	-1.5459
GO:0044271	cellular nitrogen compound biosynthetic process	-1.5168
GO:0009308	amine metabolic process	-1.5105
GO:0048584	positive regulation of response to stimulus	-1.4705
GO:0000096	sulfur amino acid metabolic process	-1.4569
GO:0044237	cellular metabolic process	-1.4453
GO:0009408	response to heat	-1.4438
GO:0022613	ribonucleoprotein complex biogenesis	-1.4393
GO:0006082	organic acid metabolic process	-1.3438
GO:0043933	macromolecular complex subunit organization	-1.3413
GO:0044283	small molecule biosynthetic process	-1.3339
GO:0051707	response to other organism	-1.3309
GO:0022607	cellular component assembly	-1.3194
GO:0042180	cellular ketone metabolic process	-1.3170
GO:0032991	macromolecular complex	-1.3135

Table S5

A

Term ID	Description	$\log_{10}$ p
GO:0001320	age-dependent response to reactive oxygen species involved in chronological cell aging	-4.5778
GO:0000466	maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	-3.5841
GO:0042254	ribosome biogenesis	-3.5668
GO:0051536	iron-sulfur cluster binding	-3.4581
GO:0051540	metal cluster binding	-3.4581
GO:0030684	preribosome	-3.3557
GO:0006882	cellular zinc ion homeostasis	-3.2627
GO:0004784	superoxide dismutase activity	-3.1392
GO:0016721	oxidoreductase activity, acting on superoxide radicals as acceptor	-3.1392

GO:0006801	superoxide metabolic process	-2.9360
GO:0016072	rRNA metabolic process	-2.6898
GO:0000469	cleavage involved in rRNA processing	-2.5947
GO:0072593	reactive oxygen species metabolic process	-2.5185
GO:0009277	fungal-type cell wall	-2.4680
GO:0006099	tricarboxylic acid cycle	-2.4656
GO:0009109	coenzyme catabolic process	-2.4656
GO:0005618	cell wall	-2.4552
GO:0030312	external encapsulating structure	-2.4424
GO:0051187	cofactor catabolic process	-2.4159
GO:0030522	intracellular receptor signaling pathway	-2.2823
GO:0030518	intracellular steroid hormone receptor signaling pathway	-2.2823
GO:0031347	regulation of defense response	-2.1333
GO:0051704	multi-organism process	-2.1088
GO:0051817	modification of morphology or physiology of other organism involved in symbiotic interaction	-2.1000
GO:0035821	modification of morphology or physiology of other organism	-2.1000
GO:0000966	RNA 5'-end processing	-2.0680
GO:0000967	rRNA 5'-end processing	-2.0680
GO:0016209	antioxidant activity	-2.0680
GO:0044085	cellular component biogenesis	-2.0572
GO:0090305	nucleic acid phosphodiester bond hydrolysis	-2.0473
GO:0015075	ion transmembrane transporter activity	-2.0116
GO:0008334	histone mRNA metabolic process	-1.9823
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	-1.9823
GO:0042868	antisense RNA metabolic process	-1.9823
GO:0071044	histone mRNA catabolic process	-1.9823
GO:0071041	antisense RNA transcript catabolic process	-1.9823
GO:0003994	aconitate hydratase activity	-1.9823
GO:0015306	sialate:cation symporter activity	-1.9823
GO:0006084	acetyl-CoA metabolic process	-1.9790
GO:0051287	NAD binding	-1.9515
GO:0005746	mitochondrial respiratory chain	-1.9249
GO:0034220	ion transmembrane transport	-1.9146
GO:0055114	oxidation-reduction process	-1.8996
GO:0009408	response to heat	-1.8991
GO:0070469	respiratory chain	-1.8991
GO:0005730	nucleolus	-1.8811
GO:0000390	spliceosomal complex disassembly	-1.8074
GO:0016076	snRNA catabolic process	-1.8074
GO:0016077	snoRNA catabolic process	-1.8074
GO:0071049	nuclear retention of pre-mRNA with aberrant 3'-ends at the site of transcription	-1.8074
GO:0071014	post-mRNA release spliceosomal complex	-1.8074
GO:0034457	Mpp10 complex	-1.8074
GO:0005749	mitochondrial respiratory chain complex II, succinate dehydrogenase complex (ubiquinone)	-1.8074
GO:0055085	transmembrane transport	-1.7831
GO:0030445	yeast-form cell wall	-1.7604
GO:0044429	mitochondrial part	-1.7493
GO:0016491	oxidoreductase activity	-1.7257

GO:0034660	ncRNA metabolic process	-1.7153
GO:0009986	cell surface	-1.7048
GO:0071944	cell periphery	-1.7036
GO:0006811	ion transport	-1.6977
GO:0065008	regulation of biological quality	-1.6876
GO:0071577	zinc II ion transmembrane transport	-1.6835
GO:0051090	regulation of sequence-specific DNA binding transcription factor activity	-1.6835
GO:0034473	U1 snRNA 3'-end processing	-1.6835
GO:0006829	zinc II ion transport	-1.6835
GO:0005385	zinc ion transmembrane transporter activity	-1.6835
GO:0008177	succinate dehydrogenase (ubiquinone) activity	-1.6835
GO:0051707	response to other organism	-1.6801
GO:0007568	aging	-1.6076
GO:0006616	SRP-dependent cotranslational protein targeting to membrane, translocation	-1.5877
GO:0032988	ribonucleoprotein complex disassembly	-1.5877
GO:0016635	oxidoreductase activity, acting on the CH-CH group of donors, quinone or related compound as acceptor	-1.5877
GO:0009636	response to toxic substance	-1.5096
GO:0006091	generation of precursor metabolites and energy	-1.4951
GO:0030446	hyphal cell wall	-1.4665
GO:0071043	CUT metabolic process	-1.4438
GO:0071034	CUT catabolic process	-1.4438
GO:0000104	succinate dehydrogenase activity	-1.4438
GO:0006396	RNA processing	-1.4305
GO:0015291	secondary active transmembrane transporter activity	-1.3986
GO:0000973	posttranscriptional tethering of RNA polymerase II gene DNA at nuclear periphery	-1.3869
GO:0030964	NADH dehydrogenase complex	-1.3869
GO:0010035	response to inorganic substance	-1.3486
GO:0015074	DNA integration	-1.3368
GO:0000972	transcription-dependent tethering of RNA polymerase II gene DNA at nuclear periphery	-1.3368
GO:0005315	inorganic phosphate transmembrane transporter activity	-1.3368
GO:0005310	dicarboxylic acid transmembrane transporter activity	-1.3368
GO:0009607	response to biotic stimulus	-1.3366
GO:0005886	plasma membrane	-1.3121
GO:0051082	unfolded protein binding	-1.3019
GO:0035435	phosphate ion transmembrane transport	-1.2922
GO:0006835	dicarboxylic acid transport	-1.2922
GO:0015114	phosphate ion transmembrane transporter activity	-1.2922
GO:0042592	homeostatic process	-1.2756
GO:0044422	organelle part	-1.2661
GO:0044446	intracellular organelle part	-1.2661

B

Term ID	Description	$\log_{10}$ p
GO:0044283	small molecule biosynthetic process	-9.7916
GO:0042180	cellular ketone metabolic process	-6.8261
GO:0044281	small molecule metabolic process	-6.2793

GO:0006082	organic acid metabolic process	-6.1367
GO:0044249	cellular biosynthetic process	-6.0667
GO:0009058	biosynthetic process	-5.9247
GO:0046394	carboxylic acid biosynthetic process	-5.5003
GO:0044271	cellular nitrogen compound biosynthetic process	-5.4159
GO:0005737	cytoplasm	-4.6465
GO:0043226	organelle	-4.4341
GO:0043229	intracellular organelle	-4.4341
GO:0005835	fatty acid synthase complex	-4.2123
GO:0032787	monocarboxylic acid metabolic process	-4.1969
GO:0016491	oxidoreductase activity	-4.1716
GO:0006631	fatty acid metabolic process	-4.0738
GO:0055114	oxidation-reduction process	-4.0521
GO:0044424	intracellular part	-4.0331
GO:0009987	cellular process	-4.0317
GO:0005623	cell	-3.9907
GO:0044464	cell part	-3.9907
GO:0008152	metabolic process	-3.7875
GO:0005622	intracellular	-3.7112
GO:0044444	cytoplasmic part	-3.6765
GO:0044445	cytosolic part	-3.5591
GO:0005829	cytosol	-3.5351
GO:0044237	cellular metabolic process	-3.5167
GO:0009309	amine biosynthetic process	-3.4213
GO:0003824	catalytic activity	-3.4015
GO:0006629	lipid metabolic process	-3.3101
GO:0004312	fatty acid synthase activity	-3.2189
GO:0070006	metalloaminopeptidase activity	-3.2189
GO:0006696	ergosterol biosynthetic process	-3.1320
GO:0050662	coenzyme binding	-3.1216
GO:0044238	primary metabolic process	-3.0973
GO:0051188	cofactor biosynthetic process	-3.0466
GO:0005811	lipid particle	-2.9725
GO:0004497	monooxygenase activity	-2.9725
GO:0005739	mitochondrion	-2.9104
GO:0043231	intracellular membrane-bounded organelle	-2.9009
GO:0006766	vitamin metabolic process	-2.7065
GO:0008237	metallopeptidase activity	-2.7065
GO:0000041	transition metal ion transport	-2.5579
GO:0008202	steroid metabolic process	-2.5236
GO:0006825	copper ion transport	-2.4918
GO:0016709	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen	-2.4918
GO:0016746	transferase activity, transferring acyl groups	-2.3955
GO:0009308	amine metabolic process	-2.3946
GO:0005740	mitochondrial envelope	-2.3762
GO:0048037	cofactor binding	-2.3445
GO:0016884	carbon-nitrogen ligase activity, with glutamine as amido-N-donor	-2.2797
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	-2.2544

GO:0018130	heterocycle biosynthetic process	-2.1875
GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups	-2.1869
GO:0051186	cofactor metabolic process	-2.1637
GO:0000786	nucleosome	-2.1118
GO:0032445	fructose import	-2.1018
GO:0032055	negative regulation of translation in response to stress	-2.1018
GO:0008686	3,4-dihydroxy-2-butanone-4-phosphate synthase activity	-2.1018
GO:0003838	sterol 24-C-methyltransferase activity	-2.1018
GO:0004647	phosphoserine phosphatase activity	-2.1018
GO:0003842	1-pyrroline-5-carboxylate dehydrogenase activity	-2.1018
GO:0004733	pyridoxamine-phosphate oxidase activity	-2.1018
GO:0004321	fatty-acyl-CoA synthase activity	-2.1018
GO:0004421	hydroxymethylglutaryl-CoA synthase activity	-2.1018
GO:0004414	homoserine O-acetyltransferase activity	-2.1018
GO:0004315	3-oxoacyl-[acyl-carrier-protein] synthase activity	-2.1018
GO:0004502	kynurenine 3-monooxygenase activity	-2.1018
GO:0015087	cobalt ion transmembrane transporter activity	-2.1018
GO:0004506	squalene monooxygenase activity	-2.1018
GO:0004317	3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity	-2.1018
GO:0004313	[acyl-carrier-protein] S-acetyltransferase activity	-2.1018
GO:0004319	enoyl-[acyl-carrier-protein] reductase (NADPH, B-specific) activity	-2.1018
GO:0071944	cell periphery	-2.0502
GO:0046915	transition metal ion transmembrane transporter activity	-2.0167
GO:0003735	structural constituent of ribosome	-2.0070
GO:0065004	protein-DNA complex assembly	-1.9958
GO:0005886	plasma membrane	-1.9843
GO:0015935	small ribosomal subunit	-1.9350
GO:0005507	copper ion binding	-1.8926
GO:0071824	protein-DNA complex subunit organization	-1.8409
GO:0031975	envelope	-1.8252
GO:0030001	metal ion transport	-1.8231
GO:0007090	(obsolete) regulation of S phase of mitotic cell cycle	-1.8025
GO:0020028	hemoglobin import	-1.8025
GO:0070681	glutaminyl-tRNAGln biosynthesis *via* transamidation	-1.8025
GO:0000461	endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	-1.8025
GO:0010133	proline catabolic process to glutamate	-1.8025
GO:0030956	glutamyl-tRNA(Gln) amidotransferase complex	-1.8025
GO:0003849	3-deoxy-7-phosphoheptulonate synthase activity	-1.8025
GO:0016208	AMP binding	-1.8025
GO:0019171	3-hydroxyacyl-[acyl-carrier-protein] dehydratase activity	-1.8025
GO:0003987	acetate-CoA ligase activity	-1.8025
GO:0008553	hydrogen-exporting ATPase activity, phosphorylative mechanism	-1.8025
GO:0016420	malonyltransferase activity	-1.8025
GO:0004035	alkaline phosphatase activity	-1.8025
GO:0015088	copper uptake transmembrane transporter activity	-1.8025
GO:0050567	glutaminyl-tRNA synthase (glutamine-hydrolyzing) activity	-1.8025
GO:0016418	S-acetyltransferase activity	-1.8025
GO:0046148	pigment biosynthetic process	-1.7531

GO:0042440	pigment metabolic process	-1.7218
GO:0035770	ribonucleoprotein granule	-1.7218
GO:0033261	(obsolete) regulation of S phase	-1.6281
GO:0006564	L-serine biosynthetic process	-1.6281
GO:0046323	glucose import	-1.6281
GO:0008301	DNA binding, bending	-1.6281
GO:0008897	holo-[acyl-carrier-protein] synthase activity	-1.6281
GO:0043813	phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity	-1.6281
GO:0016880	acid-ammonia (or amide) ligase activity	-1.6281
GO:0001302	replicative cell aging	-1.6084
GO:0055080	cation homeostasis	-1.6023
GO:0043228	non-membrane-bounded organelle	-1.5621
GO:0051028	mRNA transport	-1.5334
GO:0010696	positive regulation of spindle pole body separation	-1.5048
GO:0034729	histone H3-K79 methylation	-1.5048
GO:0043555	regulation of translation in response to stress	-1.5048
GO:0015677	copper ion import	-1.5048
GO:0070772	PAS complex	-1.5048
GO:0008169	C-methyltransferase activity	-1.5048
GO:0005198	structural molecule activity	-1.5010
GO:0034641	cellular nitrogen compound metabolic process	-1.4780
GO:0065008	regulation of biological quality	-1.4771
GO:0006325	chromatin organization	-1.4753
GO:0016791	phosphatase activity	-1.4707
GO:0005506	iron ion binding	-1.4347
GO:0006352	DNA-templated transcription, initiation	-1.4233
GO:0009066	aspartate family amino acid metabolic process	-1.4233
GO:0006807	nitrogen compound metabolic process	-1.4145
GO:0046872	metal ion binding	-1.4116
GO:0016311	dephosphorylation	-1.4114
GO:0006000	fructose metabolic process	-1.4096
GO:0046015	regulation of transcription by glucose	-1.4096
GO:0006083	acetate metabolic process	-1.4096
GO:0016653	oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor	-1.4096
GO:0004396	hexokinase activity	-1.4096
GO:0016408	C-acyltransferase activity	-1.4096
GO:0016879	ligase activity, forming carbon-nitrogen bonds	-1.4000
GO:0042592	homeostatic process	-1.3986
GO:0005840	ribosome	-1.3978
GO:0032502	developmental process	-1.3349
GO:0034627	'de novo' NAD biosynthetic process	-1.3321
GO:0043171	peptide catabolic process	-1.3321
GO:0006085	acetyl-CoA biosynthetic process	-1.3321
GO:0005678	(obsolete) chromatin assembly complex	-1.3321
GO:0005851	eukaryotic translation initiation factor 2B complex	-1.3321
GO:0006323	DNA packaging	-1.3280
GO:0051276	chromosome organization	-1.3153
GO:0043167	ion binding	-1.3098

GO:0043169	cation binding	-1.3098
GO:0034654	nucleobase-containing compound biosynthetic process	-1.2832
GO:0007568	aging	-1.2766
GO:0006333	chromatin assembly or disassembly	-1.2766
GO:0032993	protein-DNA complex	-1.2766
GO:0046856	phosphatidylinositol dephosphorylation	-1.2668
GO:0006560	proline metabolic process	-1.2668
GO:0006013	mannose metabolic process	-1.2668
GO:0030287	cell wall-bounded periplasmic space	-1.2668
GO:0042597	periplasmic space	-1.2668
GO:0016877	ligase activity, forming carbon-sulfur bonds	-1.2668

Table S6

A

Term ID	Description	$\log_{10}$ p
GO:0010466	negative regulation of peptidase activity	-3.8118
GO:0030414	peptidase inhibitor activity	-3.8118
GO:0030162	regulation of proteolysis	-3.5399
GO:0061134	peptidase regulator activity	-3.0407
GO:0000324	fungal-type vacuole	-2.8544
GO:0051346	negative regulation of hydrolase activity	-2.8224
GO:0005773	vacuole	-2.6756
GO:0030446	hyphal cell wall	-2.2946
GO:0006536	glutamate metabolic process	-2.2802
GO:0044092	negative regulation of molecular function	-2.2230
GO:0006098	pentose-phosphate shunt	-2.1868
GO:0030001	metal ion transport	-2.0426
GO:0072509	divalent inorganic cation transmembrane transporter activity	-2.0275
GO:0044419	interspecies interaction between organisms	-2.0258
GO:0048037	cofactor binding	-1.9288
GO:0046677	response to antibiotic	-1.9032
GO:0071497	cellular response to freezing	-1.9032
GO:0006833	water transport	-1.9032
GO:0016246	RNA interference	-1.9032
GO:0002921	negative regulation of humoral immune response	-1.9032
GO:0042044	fluid transport	-1.9032
GO:0071507	MAPK cascade involved in conjugation with cellular fusion	-1.9032
GO:0002673	regulation of acute inflammatory response	-1.9032
GO:0042784	active evasion of host immune response *via* regulation of host complement system	-1.9032
GO:0043418	homocysteine catabolic process	-1.9032
GO:2000257	regulation of protein activation cascade	-1.9032
GO:0070613	regulation of protein processing	-1.9032
GO:2000258	negative regulation of protein activation cascade	-1.9032
GO:0071511	inactivation of MAPK activity involved in conjugation with cellular fusion	-1.9032
GO:0032807	DNA ligase IV complex	-1.9032
GO:0003985	acetyl-CoA C-acetyltransferase activity	-1.9032

GO:0005178	integrin binding	-1.9032
GO:0004345	glucose-6-phosphate dehydrogenase activity	-1.9032
GO:0004351	glutamate decarboxylase activity	-1.9032
GO:0008420	CTD phosphatase activity	-1.9032
GO:0008466	glycogenin glucosyltransferase activity	-1.9032
GO:0016040	glutamate synthase (NADH) activity	-1.9032
GO:0016532	superoxide dismutase copper chaperone activity	-1.9032
GO:0070551	endoribonuclease activity, cleaving siRNA-paired mRNA	-1.9032
GO:0005372	water transmembrane transporter activity	-1.9032
GO:0015250	water channel activity	-1.9032
GO:0070051	fibrinogen binding	-1.9032
GO:0004347	glucose-6-phosphate isomerase activity	-1.9032
GO:0016860	intramolecular oxidoreductase activity	-1.8950
GO:0004857	enzyme inhibitor activity	-1.8950
GO:0007155	cell adhesion	-1.8464
GO:0072503	cellular divalent inorganic cation homeostasis	-1.7819
GO:0072507	divalent inorganic cation homeostasis	-1.7819
GO:0030312	external encapsulating structure	-1.7342
GO:0070838	divalent metal ion transport	-1.6834
GO:0006508	proteolysis	-1.6807
GO:0051246	regulation of protein metabolic process	-1.6284
GO:0006266	DNA ligation	-1.6048
GO:0046938	phytochelatin biosynthetic process	-1.6048
GO:0070940	dephosphorylation of RNA polymerase II C-terminal domain	-1.6048
GO:0051103	DNA ligation involved in DNA repair	-1.6048
GO:0051792	medium-chain fatty acid biosynthetic process	-1.6048
GO:0016441	posttranscriptional gene silencing	-1.6048
GO:0051791	medium-chain fatty acid metabolic process	-1.6048
GO:0003910	DNA ligase (ATP) activity	-1.6048
GO:0004573	mannosyl-oligosaccharide glucosidase activity	-1.6048
GO:0004333	fumarate hydratase activity	-1.6048
GO:0015369	calcium:proton antiporter activity	-1.6048
GO:0072511	divalent inorganic cation transport	-1.5563
GO:0050662	coenzyme binding	-1.5193
GO:0042144	vacuole fusion, non-autophagic	-1.4824
GO:0071470	cellular response to osmotic stress	-1.4481
GO:0008236	serine-type peptidase activity	-1.4481
GO:0032101	regulation of response to external stimulus	-1.4314
GO:0015680	intracellular copper ion transport	-1.4314
GO:0006106	fumarate metabolic process	-1.4314
GO:0000111	nucleotide-excision repair factor 2 complex	-1.4314
GO:0032934	sterol binding	-1.4314
GO:0005516	calmodulin binding	-1.4314
GO:0004030	aldehyde dehydrogenase [NAD(P)+] activity	-1.4314
GO:0032403	protein complex binding	-1.4314
GO:0016886	ligase activity, forming phosphoric ester bonds	-1.4314
GO:0009986	cell surface	-1.4078
GO:0051703	intraspecies interaction between organisms	-1.3840

GO:0044011	single-species biofilm formation on inanimate substrate	-1.3840
GO:0035821	modification of morphology or physiology of other organism	-1.3840
GO:0042277	peptide binding	-1.3840
GO:0017171	serine hydrolase activity	-1.3840
GO:0008238	exopeptidase activity	-1.3541
GO:0048583	regulation of response to stimulus	-1.3532
GO:0010605	negative regulation of macromolecule metabolic process	-1.3275
GO:0001411	hyphal tip	-1.3253
GO:0043043	peptide biosynthetic process	-1.3091
GO:0006995	cellular response to nitrogen starvation	-1.3091
GO:0043562	cellular response to nitrogen levels	-1.3091
GO:0072488	ammonium transmembrane transport	-1.3091
GO:0045239	tricarboxylic acid cycle enzyme complex	-1.3091
GO:0019003	GDP binding	-1.3091
GO:0008519	ammonium transmembrane transporter activity	-1.3091
GO:0071900	regulation of protein serine/threonine kinase activity	-1.2977
GO:0022610	biological adhesion	-1.2776
GO:0009064	glutamine family amino acid metabolic process	-1.2712

B

Term ID	Description	$\log_{10}$ p
GO:0070911	global genome nucleotide-excision repair	-5.0043
GO:0051784	negative regulation of nuclear division	-3.6660
GO:0031577	spindle checkpoint	-3.5274
GO:0000786	nucleosome	-3.3543
GO:0009303	rRNA transcription	-3.1674
GO:0045786	negative regulation of cell cycle	-3.0501
GO:0031298	replication fork protection complex	-2.9471
GO:0051129	negative regulation of cellular component organization	-2.8553
GO:0006325	chromatin organization	-2.5680
GO:0006289	nucleotide-excision repair	-2.5643
GO:0006323	DNA packaging	-2.5243
GO:0006333	chromatin assembly or disassembly	-2.4677
GO:0032993	protein-DNA complex	-2.4677
GO:0005657	replication fork	-2.4497
GO:0034728	nucleosome organization	-2.3651
GO:0043935	sexual sporulation resulting in formation of a cellular spore	-2.3182
GO:0050794	regulation of cellular process	-2.2819
GO:0071103	DNA conformation change	-2.2738
GO:0000916	actomyosin contractile ring contraction	-2.2452
GO:0005971	ribonucleoside-diphosphate reductase complex	-2.2452
GO:0071824	protein-DNA complex subunit organization	-2.1917
GO:0042788	polysomal ribosome	-2.1207
GO:0004100	chitin synthase activity	-2.1207
GO:0005089	Rho guanyl-nucleotide exchange factor activity	-2.1207
GO:0004748	ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor	-2.1207

GO:0016725	oxidoreductase activity, acting on CH or CH2 groups	-2.1207
GO:0050789	regulation of biological process	-2.0643
GO:0022402	cell cycle process	-2.0554
GO:0009263	deoxyribonucleotide biosynthetic process	-2.0241
GO:0005935	cellular bud neck	-2.0066
GO:0051276	chromosome organization	-1.9724
GO:0007049	cell cycle	-1.9583
GO:0000785	chromatin	-1.8737
GO:0006259	DNA metabolic process	-1.8483
GO:0000083	regulation of transcription involved in G1/S transition of mitotic cell cycle	-1.8212
GO:0009262	deoxyribonucleotide metabolic process	-1.8212
GO:0065007	biological regulation	-1.8081
GO:0005933	cellular bud	-1.7677
GO:0048610	(obsolete) cellular process involved in reproduction	-1.7233
GO:0051726	regulation of cell cycle	-1.7020
GO:0051716	cellular response to stimulus	-1.6839
GO:0051128	regulation of cellular component organization	-1.6813
GO:0022607	cellular component assembly	-1.6612
GO:0035023	regulation of Rho protein signal transduction	-1.6466
GO:0004407	histone deacetylase activity	-1.6466
GO:0019213	deacetylase activity	-1.5804
GO:0030427	site of polarized growth	-1.5560
GO:0070592	cell wall polysaccharide biosynthetic process	-1.5508
GO:0006022	aminoglycan metabolic process	-1.5508
GO:0034645	cellular macromolecule biosynthetic process	-1.5428
GO:0009059	macromolecule biosynthetic process	-1.5358
GO:0044249	cellular biosynthetic process	-1.5020
GO:0009058	biosynthetic process	-1.4706
GO:0016575	histone deacetylation	-1.4496
GO:0030428	cell septum	-1.4496
GO:0000003	reproduction	-1.4194
GO:0032502	developmental process	-1.3913
GO:0008194	UDP-glycosyltransferase activity	-1.3682
GO:0043228	non-membrane-bounded organelle	-1.3674
GO:0043232	intracellular non-membrane-bounded organelle	-1.3674
GO:0007165	signal transduction	-1.3641
GO:0023052	signaling	-1.3597
GO:0005844	polysome	-1.3501
GO:0022403	cell cycle phase	-1.3211
GO:0000910	cytokinesis	-1.3161
GO:0070589	cellular component macromolecule biosynthetic process	-1.3000
GO:0006633	fatty acid biosynthetic process	-1.2698
GO:0006974	cellular response to DNA damage stimulus	-1.2649

Table S7

A

Term ID	Description	log10 p
GO:0000302	response to reactive oxygen species	-4.3669
GO:0030446	hyphal cell wall	-4.2699
GO:0005829	cytosol	-3.2834
GO:0006979	response to oxidative stress	-2.9815
GO:0006083	acetate metabolic process	-2.8445
GO:0055114	oxidation-reduction process	-2.6230
GO:0051704	multi-organism process	-2.5662
GO:0007571	age-dependent general metabolic decline	-2.5292
GO:0006882	cellular zinc ion homeostasis	-2.5292
GO:0019725	cellular homeostasis	-2.4860
GO:0030312	external encapsulating structure	-2.4327
GO:0016491	oxidoreductase activity	-2.3912
GO:0045454	cell redox homeostasis	-2.2960
GO:0010035	response to inorganic substance	-2.1045
GO:0044419	interspecies interaction between organisms	-2.0718
GO:0022610	biological adhesion	-2.0362
GO:0008289	lipid binding	-1.9714
GO:0042710	biofilm formation	-1.9300
GO:0061077	chaperone-mediated protein folding	-1.9143
GO:0000448	cleavage in ITS2 between 5.8S rRNA and LSU-rRNA of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	-1.9143
GO:0006833	water transport	-1.9143
GO:0070613	regulation of protein processing	-1.9143
GO:0032780	negative regulation of ATPase activity	-1.9143
GO:0002921	negative regulation of humoral immune response	-1.9143
GO:0070370	cellular heat acclimation	-1.9143
GO:0042044	fluid transport	-1.9143
GO:0070413	trehalose metabolism in response to stress	-1.9143
GO:0006451	translational readthrough	-1.9143
GO:0042450	arginine biosynthetic process *via* ornithine	-1.9143
GO:0006295	nucleotide-excision repair, DNA incision, 3'-to lesion	-1.9143
GO:0002673	regulation of acute inflammatory response	-1.9143
GO:0042784	active evasion of host immune response *via* regulation of host complement system	-1.9143
GO:0019413	acetate biosynthetic process	-1.9143
GO:0010286	heat acclimation	-1.9143
GO:0071497	cellular response to freezing	-1.9143
GO:0051084	'de novo' posttranslational protein folding	-1.9143
GO:0070389	chaperone cofactor-dependent protein refolding	-1.9143
GO:2000257	regulation of protein activation cascade	-1.9143
GO:2000258	negative regulation of protein activation cascade	-1.9143
GO:0051085	chaperone mediated protein folding requiring cofactor	-1.9143
GO:0004056	argininosuccinate lyase activity	-1.9143
GO:0015250	water channel activity	-1.9143
GO:0005178	integrin binding	-1.9143
GO:0043531	ADP binding	-1.9143

GO:0004657	proline dehydrogenase activity	-1.9143
GO:0000285	1-phosphatidylinositol-3-phosphate 5-kinase activity	-1.9143
GO:0004619	phosphoglycerate mutase activity	-1.9143
GO:0017091	AU-rich element binding	-1.9143
GO:0003960	NADPH:quinone reductase activity	-1.9143
GO:0004345	glucose-6-phosphate dehydrogenase activity	-1.9143
GO:0032440	2-alkenal reductase [NAD(P)] activity	-1.9143
GO:0005372	water transmembrane transporter activity	-1.9143
GO:0070051	fibrinogen binding	-1.9143
GO:0007155	cell adhesion	-1.8763
GO:0072593	reactive oxygen species metabolic process	-1.8030
GO:0072507	divalent inorganic cation homeostasis	-1.8030
GO:0007568	aging	-1.7167
GO:0004520	endodeoxyribonuclease activity	-1.7043
GO:0009084	glutamine family amino acid biosynthetic process	-1.6594
GO:0006950	response to stress	-1.6451
GO:0032787	monocarboxylic acid metabolic process	-1.6422
GO:0051469	vesicle fusion with vacuole	-1.6159
GO:0009051	pentose-phosphate shunt, oxidative branch	-1.6159
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	-1.6159
GO:0042262	DNA protection	-1.6159
GO:0033194	response to hydroperoxide	-1.6159
GO:0010133	proline catabolic process to glutamate	-1.6159
GO:0003987	acetate-CoA ligase activity	-1.6159
GO:0016812	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides	-1.6159
GO:0016208	AMP binding	-1.6159
GO:0016307	phosphatidylinositol phosphate kinase activity	-1.6159
GO:0005625	(obsolete) soluble fraction	-1.5505
GO:0032266	phosphatidylinositol-3-phosphate binding	-1.5390
GO:0004536	deoxyribonuclease activity	-1.5390
GO:0019318	hexose metabolic process	-1.5175
GO:0065008	regulation of biological quality	-1.4827
GO:0007033	vacuole organization	-1.4796
GO:0009986	cell surface	-1.4545
GO:0043335	protein unfolding	-1.4424
GO:0060988	lipid tube assembly	-1.4424
GO:0032101	regulation of response to external stimulus	-1.4424
GO:0005769	early endosome	-1.4424
GO:0034657	GID complex	-1.4424
GO:0004559	alpha-mannosidase activity	-1.4424
GO:0004030	aldehyde dehydrogenase [NAD(P)+] activity	-1.4424
GO:0032403	protein complex binding	-1.4424
GO:0016880	acid-ammonia (or amide) ligase activity	-1.4424
GO:0032934	sterol binding	-1.4424
GO:0006623	protein targeting to vacuole	-1.4409
GO:0072665	protein localization to vacuole	-1.4409
GO:0051703	intraspecies interaction between organisms	-1.4043
GO:0044011	single-species biofilm formation on inanimate substrate	-1.4043

GO:0009628	response to abiotic stimulus	-1.3752
GO:0016209	antioxidant activity	-1.3742
GO:0016052	carbohydrate catabolic process	-1.3703
GO:0009311	oligosaccharide metabolic process	-1.3454
GO:0042743	hydrogen peroxide metabolic process	-1.3201
GO:0071577	zinc II ion transmembrane transport	-1.3201
GO:0008105	asymmetric protein localization	-1.3201
GO:0008272	sulfate transport	-1.3201
GO:0051090	regulation of sequence-specific DNA binding transcription factor activity	-1.3201
GO:0070772	PAS complex	-1.3201
GO:0004792	thiosulfate sulfurtransferase activity	-1.3201
GO:0005385	zinc ion transmembrane transporter activity	-1.3201
GO:0018456	aryl-alcohol dehydrogenase (NAD+) activity	-1.3201
GO:0009064	glutamine family amino acid metabolic process	-1.2911
GO:0051082	unfolded protein binding	-1.2893
GO:0030258	lipid modification	-1.2655

B

Term ID	Description	log10 p
GO:0006323	DNA packaging	-12,25
GO:0006520	cellular amino acid metabolic process	-11,96
GO:0009308	amine metabolic process	-11,89
GO:0044106	cellular amine metabolic process	-11,49
GO:0065004	protein-DNA complex assembly	-10,92
GO:0071103	DNA conformation change	-10,74
GO:0046417	chorismate metabolic process	-10,70
GO:0009073	aromatic amino acid family biosynthetic process	-10,70
GO:0009072	aromatic amino acid family metabolic process	-10,48
GO:0006333	chromatin assembly or disassembly	-10,40
GO:0032993	protein-DNA complex	-10,40
GO:0071824	protein-DNA complex subunit organization	-10,26
GO:0031298	replication fork protection complex	-9,65
GO:0019438	aromatic compound biosynthetic process	-9,51
GO:0000786	nucleosome	-8,72
GO:0043648	dicarboxylic acid metabolic process	-8,44
GO:0000785	chromatin	-8,43
GO:0044281	small molecule metabolic process	-8,38
GO:0044283	small molecule biosynthetic process	-8,34
GO:0005657	replication fork	-7,50
GO:0006725	cellular aromatic compound metabolic process	-7,48
GO:0051276	chromosome organization	-7,32
GO:0044271	cellular nitrogen compound biosynthetic process	-7,21
GO:0016833	oxo-acid-lyase activity	-6,74
GO:0005694	chromosome	-6,31
GO:0009058	biosynthetic process	-6,25
GO:0006082	organic acid metabolic process	-6,13

GO:0070911	global genome nucleotide-excision repair	-5,92
GO:0022607	cellular component assembly	-5,82
GO:0042180	cellular ketone metabolic process	-5,82
GO:0043935	sexual sporulation resulting in formation of a cellular spore	-5,73
GO:0042430	indole-containing compound metabolic process	-5,61
GO:0044249	cellular biosynthetic process	-5,35
GO:0034729	histone H3-K79 methylation	-5,33
GO:0034622	cellular macromolecular complex assembly	-5,16
GO:0006807	nitrogen compound metabolic process	-5,05
GO:0008152	metabolic process	-4,95
GO:0034641	cellular nitrogen compound metabolic process	-4,72
GO:0048610	(obsolete) cellular process involved in reproduction	-4,58
GO:0016829	lyase activity	-4,58
GO:0044238	primary metabolic process	-4,07
GO:0005950	anthranilate synthase complex	-3,94
GO:0003849	3-deoxy-7-phosphoheptulonate synthase activity	-3,94
GO:0043933	macromolecular complex subunit organization	-3,87
GO:0006081	cellular aldehyde metabolic process	-3,87
GO:0016830	carbon-carbon lyase activity	-3,69
GO:0051784	negative regulation of nuclear division	-3,61
GO:0008301	DNA binding, bending	-3,47
GO:0019202	amino acid kinase activity	-3,47
GO:0004049	anthranilate synthase activity	-3,47
GO:0044085	cellular component biogenesis	-3,45
GO:0000003	reproduction	-3,43
GO:0003824	catalytic activity	-3,41
GO:0031577	spindle checkpoint	-3,40
GO:0032502	developmental process	-3,33
GO:0046487	glyoxylate metabolic process	-3,17
GO:0009088	threonine biosynthetic process	-3,17
GO:0006352	DNA-templated transcription, initiation	-3,12
GO:0044237	cellular metabolic process	-3,09
GO:0003677	DNA binding	-3,00
GO:0018130	heterocycle biosynthetic process	-2,96
GO:0004568	chitinase activity	-2,95
GO:0009986	cell surface	-2,88
GO:0009303	rRNA transcription	-2,86
GO:0005488	binding	-2,84
GO:0000947	amino acid catabolic process to alcohol *via* Ehrlich pathway	-2,78
GO:0043228	non-membrane-bounded organelle	-2,77
GO:0043232	intracellular non-membrane-bounded organelle	-2,77
GO:0009277	fungal-type cell wall	-2,74
GO:0030312	external encapsulating structure	-2,71
GO:0045786	negative regulation of cell cycle	-2,69
GO:0009092	homoserine metabolic process	-2,64
GO:0006566	threonine metabolic process	-2,64
GO:0031981	nuclear lumen	-2,61
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	-2,50

GO:0000105	histidine biosynthetic process	-2,41
GO:0051129	negative regulation of cellular component organization	-2,41
GO:0009069	serine family amino acid metabolic process	-2,37
GO:0051287	NAD binding	-2,37
GO:0009987	cellular process	-2,33
GO:0008213	protein alkylation	-2,33
GO:0000096	sulfur amino acid metabolic process	-2,33
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	-2,32
GO:0006996	organelle organization	-2,32
GO:0005975	carbohydrate metabolic process	-2,26
GO:0005634	nucleus	-2,18
GO:0051726	regulation of cell cycle	-2,09
GO:0016740	transferase activity	-2,08
GO:0006325	chromatin organization	-2,07
GO:0006301	postreplication repair	-2,02
GO:0016763	transferase activity, transferring pentosyl groups	-2,02
GO:0006281	DNA repair	-1,98
GO:0016569	covalent chromatin modification	-1,98
GO:0000066	mitochondrial ornithine transport	-1,97
GO:0070194	synaptonemal complex disassembly	-1,97
GO:0006438	valyl-tRNA aminoacylation	-1,97
GO:0000212	meiotic spindle organization	-1,97
GO:0015822	ornithine transport	-1,97
GO:0006069	ethanol oxidation	-1,97
GO:0090306	spindle assembly involved in meiosis	-1,97
GO:0009436	glyoxylate catabolic process	-1,97
GO:0033859	furaldehyde metabolic process	-1,97
GO:0000064	L-ornithine transmembrane transporter activity	-1,97
GO:0004512	inositol-3-phosphate synthase activity	-1,97
GO:0004832	valine-tRNA ligase activity	-1,97
GO:0004635	phosphoribosyl-AMP cyclohydrolase activity	-1,97
GO:0004636	phosphoribosyl-ATP diphosphatase activity	-1,97
GO:0004414	homoserine O-acetyltransferase activity	-1,97
GO:0004779	sulfate adenylyltransferase activity	-1,97
GO:0004413	homoserine kinase activity	-1,97
GO:0004834	tryptophan synthase activity	-1,97
GO:0004048	anthranilate phosphoribosyltransferase activity	-1,97
GO:0004107	chorismate synthase activity	-1,97
GO:0008843	endochitinase activity	-1,97
GO:0004399	histidinol dehydrogenase activity	-1,97
GO:0047964	glyoxylate reductase activity	-1,97
GO:0051903	S-(hydroxymethyl)glutathione dehydrogenase activity	-1,97
GO:0004425	indole-3-glycerol-phosphate synthase activity	-1,97
GO:0033833	hydroxymethylfurfural reductase (NADH) activity	-1,97
GO:0044428	nuclear part	-1,94
GO:0005576	extracellular region	-1,85
GO:0070279	vitamin B6 binding	-1,81
GO:0044424	intracellular part	-1,75

GO:0048037	cofactor binding	-1,71
GO:0006974	cellular response to DNA damage stimulus	-1,68
GO:0016798	hydrolase activity, acting on glycosyl bonds	-1,68
GO:0042256	mature ribosome assembly	-1,67
GO:0060962	regulation of ribosomal protein gene transcription from RNA polymerase II promoter	-1,67
GO:0046292	formaldehyde metabolic process	-1,67
GO:0009328	phenylalanine-tRNA ligase complex	-1,67
GO:0043023	ribosomal large subunit binding	-1,67
GO:0043236	laminin binding	-1,67
GO:0050840	extracellular matrix binding	-1,67
GO:0047536	2-aminoadipate transaminase activity	-1,67
GO:0016208	AMP binding	-1,67
GO:0008113	peptide-methionine (S)-S-oxide reductase activity	-1,67
GO:0003987	acetate-CoA ligase activity	-1,67
GO:0004329	formate-tetrahydrofolate ligase activity	-1,67
GO:0016872	intramolecular lyase activity	-1,67
GO:0004123	cystathionine gamma-lyase activity	-1,67
GO:0019842	vitamin binding	-1,58
GO:0006790	sulfur compound metabolic process	-1,56
GO:0016043	cellular component organization	-1,53
GO:0043414	macromolecule methylation	-1,52
GO:0006259	DNA metabolic process	-1,52
GO:0005622	intracellular	-1,52
GO:0000910	cytokinesis	-1,50
GO:0019218	regulation of steroid metabolic process	-1,50
GO:0051304	chromosome separation	-1,50
GO:0000916	actomyosin contractile ring contraction	-1,50
GO:0010969	regulation of pheromone-dependent signal transduction involved in conjugation with cellular fusion	-1,50
GO:0051307	meiotic chromosome separation	-1,50
GO:0006432	phenylalanyl-tRNA aminoacylation	-1,50
GO:0006021	inositol biosynthetic process	-1,50
GO:0032443	regulation of ergosterol biosynthetic process	-1,50
GO:0005850	eukaryotic translation initiation factor 2 complex	-1,50
GO:0000798	nuclear cohesin complex	-1,50
GO:0008278	cohesin complex	-1,50
GO:0001968	fibronectin binding	-1,50
GO:0042602	riboflavin reductase (NADPH) activity	-1,50
GO:0003873	6-phosphofructo-2-kinase activity	-1,50
GO:0016838	carbon-oxygen lyase activity, acting on phosphates	-1,50
GO:0016880	acid-ammonia (or amide) ligase activity	-1,50
GO:0004826	phenylalanine-tRNA ligase activity	-1,50
GO:0009108	coenzyme biosynthetic process	-1,46
GO:0005623	cell	-1,42
GO:0044464	cell part	-1,42
GO:0051128	regulation of cellular component organization	-1,40
GO:0031974	membrane-enclosed lumen	-1,38
GO:0006003	fructose 2,6-bisphosphate metabolic process	-1,37
GO:0030260	entry into host cell	-1,37

GO:0070193	synaptonemal complex organization	-1,37
GO:0010696	positive regulation of spindle pole body separation	-1,37
GO:0070181	small ribosomal subunit rRNA binding	-1,37
GO:0016774	phosphotransferase activity, carboxyl group as acceptor	-1,37
GO:0004100	chitin synthase activity	-1,37
GO:0046483	heterocycle metabolic process	-1,34
GO:0071840	cellular component organization or biogenesis	-1,34
GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	-1,29
GO:0045903	positive regulation of translational fidelity	-1,28
GO:0006000	fructose metabolic process	-1,28
GO:0042816	vitamin B6 metabolic process	-1,28
GO:0006821	chloride transport	-1,28
GO:0006083	acetate metabolic process	-1,28
GO:2000241	regulation of reproductive process	-1,28
GO:0006541	glutamine metabolic process	-1,28
GO:0070898	RNA polymerase III transcriptional preinitiation complex assembly	-1,28
GO:0000400	four-way junction DNA binding	-1,28
GO:0016671	oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor	-1,28

Table S8

A

Gene name	Gene symbol	wt, 24 h	wt, 48 h	*zrt2*Δ/Δ 24 h	*zrt2*Δ/Δ 48 h
orf19.1098		5,661109	0,329129	6,264898	5,376548
orf19.2121		5,361528	0,234696	5,817350	5,060243
orf19.1097	*ALS2*	3,872757	0,001339	3,618407	2,969133
orf19.7434	*GLG2*	3,321834	1,836319	3,117793	2,363455
orf19.2038		3,293890	-0,241986	3,179431	2,822981
orf19.7385		3,204742	1,086029	2,614426	1,793247
orf19.1795	*PUF3*	3,165295	-0,687515	-1,710762	-2,283943
orf19.5561	*RAV2*	3,104699	-0,023429	3,230732	2,374538
orf19.539	*LAP3*	3,088119	-1,939316	2,926056	1,979639
orf19.6077		2,787304	0,047409	2,552734	1,699770
orf19.4980	*HSP70*	2,579918	-0,585390	2,253272	1,394795
orf19.1153	*GAD1*	2,268386	-1,676973	2,087830	1,519299
orf19.4979	*KNS1*	2,190080	1,085763	1,959414	1,245674
orf19.6257	*GLT1*	2,116703	0,485084	2,186536	1,791596
orf19.405	*VCX1*	1,993752	-0,289645	2,102148	1,478295
orf19.338		1,932078	0,106288	1,674003	0,837674
orf19.5605		1,783315	-0,852655	1,515517	1,625225
orf19.6759		1,778262	-0,382966	1,703117	0,987920
orf19.1642		1,718428	-0,769235	-0,687941	-0,165233
orf19.2865		1,712766	-0,247373	1,819065	0,928107
orf19.1610		1,711727	-1,250032	-2,081133	-0,485272
orf19.7121		1,590641	0,410724	1,176189	0,342984
orf19.5391		1,554386	0,384223	1,465945	0,585832
orf19.1636	*STE50*	1,532811	-0,430377	-0,524221	-0,186529

orf19.5365		1,520836	0,020083	1,322105	0,844380
orf19.4530.1		1,520207	-2,193027	1,557927	0,616488
orf19.5424		1,511684	0,247945	1,581330	0,656055
orf19.6066		1,509276	-0,143979	1,267365	0,621145
orf19.2049		1,508358	-0,525333	-0,455029	0,025377
orf19.2241	*PST1*	1,506988	-1,799298	1,500795	0,516978
orf19.1435	*TEF1*	1,498356	-2,092003	1,099218	0,929517
orf19.4752	*MSN4*	1,480957	-0,816024	1,302268	0,501708
orf19.1960	*CLN3*	1,476339	-1,155598	1,249180	0,172701
orf19.1183		1,438691	-1,195869	1,925814	1,200334
orf19.543	*FUM11*	1,417318	-2,466972	1,361642	0,571630
orf19.6078	*POL93*	1,414335	-2,468962	1,173228	-0,737753
orf19.5011	*KAR9*	1,390042	0,033777	1,285481	0,683168
orf19.6722		1,335214	-1,241323	1,148367	0,680773
orf19.2461	*PRN4*	1,330976	-0,215975	0,648462	0,286200
orf19.2769		1,323641	-0,501754	0,905468	0,507094
orf19.1149	*MRF1*	1,290638	-1,755243	1,190773	0,241529
orf19.1890		1,254419	0,187348	1,363704	0,680498
orf19.2018		1,221426	-1,153250	-1,147633	-0,483363
orf19.4780		1,190534	-0,060959	1,453533	0,797049
orf19.2440	*RTT101*	1,165186	-0,271309	-0,181461	-0,291041
orf19.2619	*PHO113*	1,134619	-0,311293	0,277740	-0,179386
orf19.2737		1,131440	-0,311373	1,234481	0,788006
orf19.4898		1,069990	-1,005686	1,563891	0,610137
orf19.1392		1,062750	-1,252812	1,047780	0,393219
orf19.1974	*TFS1*	1,057915	-2,352273	0,824461	0,599931
orf19.5393		1,036415	-0,536607	0,750459	0,765703
orf19.1614	*MEP1*	0,991857	-1,292350	1,314785	0,420869
orf19.3659		0,956733	-0,887727	0,422836	0,208353
orf19.2963		0,863069	-0,504989	-0,643346	-0,203417
orf19.5602	*BMT6*	0,819525	-0,512397	0,512151	-0,156379
orf19.4449		0,812899	-0,712693	1,274507	0,714813
orf19.6660		0,800967	-0,929276	0,417569	0,189879
orf19.2474	*PRC3*	0,799020	-1,442457	0,814000	0,522114
orf19.5798	*LIG4*	0,793798	-1,412098	0,425774	0,049271
orf19.5892		0,702954	-2,008088	0,943875	0,702023
orf19.1591	*ERG10*	0,558697	-1,592880	-1,640014	-1,018763
orf19.7304		0,531162	-1,167269	0,819971	0,650116
orf19.2903		0,480893	-0,870101	0,368192	0,210867
orf19.4943	*PSA2*	0,389010	-1,408503	0,511803	-0,305060
orf19.3888	*PGI1*	0,384386	-3,281943	0,417505	-0,239372
orf19.1504		0,313227	-0,727285	0,329521	-0,170731
orf19.7610	*PTP3*	0,194115	-2,258636	0,056421	-0,990949

B

Gene name	Gene symbol	wt, 24 h	wt, 48 h	*zrt2*Δ/Δ 24 h	*zrt2*Δ/Δ 48 h
orf19.1863		-3,054687	1,235171	-2,518286	-0,481647
orf19.4631	*ERG251*	-2,972433	-1,645758	-2,729997	-3,297343
orf19.5779	*RNR1*	-2,013664	-0,448132	-1,346881	-0,206870
orf19.1697		-1,547593	1,969606	-1,205076	-1,579564
orf19.5861.1		-1,538985	0,109940	-1,391335	-0,715689
orf19.1075.1		-1,391960	0,024740	-1,645681	-1,075430
orf19.2772	*HOS3*	-1,159636	3,400523	-0,349436	-0,221455
orf19.4674	*BMT9*	-0,732126	1,096383	0,143849	-0,083534
orf19.3001	*TEM1*	-0,614363	0,508288	-0,656883	-0,134967
orf19.4792		0,066990	2,028634	0,686838	0,151936
orf19.5069		0,156169	4,084521	0,485547	1,328319

Table S9

Strains	RG	SD	Strains	RG	SD	Strains	RG	SD	Strains	RG	SD	Strains	RG	SD
24h LZM starvation			48h LZM limitation			48h LZM 2nd limitation			48h LZM+ 5µM ZC			48h LZM+5µM ZnSO4		
orf19.5558	0,257	0,371	orf19.5558	0,033	0,116	orf19.454	0,191	0,253	orf19.5558	0,169	0,034	orf19.5558	0,150	0,018
orf19.7150	0,318	0,421	orf19.7150	0,061	0,106	orf19.1069	0,221	0,249	orf19.7150	0,185	0,035	orf19.7150	0,158	0,018
orf19.6124	0,604	0,519	orf19.3127	0,145	0,226	orf19.2842	0,293	0,229	orf19.6798	0,437	0,008	orf19.3794	0,593	0,448
orf19.6798	0,606	0,073	orf19.3794	0,21	0,055	orf19.2753	0,306	0,232	orf19.3794	0,470	0,265	orf19.6798	0,640	0,076
orf19.4545	0,668	0,091	orf19.2842	0,257	0,250	orf19.1499	0,309	0,246	orf19.454	0,526	0,191	orf19.6124	0,744	0,534
orf19.3063	0,685	0,213	orf19.3063	0,273	0,083	orf19.3305	0,321	0,236	orf19.6124	0,590	0,305	orf19.1187	0,840	0,016
orf19.2119	0,745	0,092	orf19.2808	0,391	0,244	orf19.3127	0,327	0,228	orf19.921	0,614	0,133	orf19.4545	0,854	0,074
orf19.1973	0,802	0,078	orf19.3625	0,405	0,251	orf19.921	0,330	0,250	orf19.3305	0,614	0,195	orf19.5249	0,869	0,041
orf19.5917	0,805	0,083	orf19.2745	0,503	0,117	orf19.3188	0,335	0,238	orf19.1499	0,620	0,225	orf19.5343	0,874	0,049
orf19.1926	0,806	0,004	orf19.6798	0,526	0,030	orf19.2730	0,344	0,243	orf19.1069	0,620	0,195	orf19.6121	0,888	0,041
orf19.4056	0,814	0,060	orf19.6985	0,563	0,183	orf19.3794	0,355	0,224	orf19.3063	0,632	0,036	orf19.6817	0,889	0,043
orf19.2745	0,816	0,154	orf19.2961	0,573	0,355	orf19.1253	0,366	0,247	orf19.2730	0,640	0,183	orf19.5729	0,896	0,036
orf19.3308	0,817	0,108	orf19.2753	0,58	0,248	orf19.2808	0,370	0,230	orf19.3190	0,641	0,143	orf19.3063	0,899	0,083
orf19.3127	0,818	0,213	orf19.1069	0,602	0,053	orf19.3625	0,389	0,225	orf19.2647	0,644	0,177	orf19.2315	0,899	0,005
orf19.5001	0,822	0,041	orf19.7371	0,605	0,193	orf19.2647	0,392	0,244	orf19.1253	0,650	0,160	orf19.6109	0,906	0,042
orf19.2476	0,844	0,147	orf19.3193	0,615	0,058	orf19.2745	0,401	0,232	orf19.2088	0,657	0,028	orf19.6680	0,906	0,046
orf19.921	0,848	0,002	orf19.2088	0,641	0,043	orf19.3063	0,406	0,228	orf19.3188	0,663	0,130	orf19.6888	0,908	0,033
orf19.3809	0,856	0,051	orf19.2612	0,642	0,276	orf19.5558	0,410	0,231	orf19.2646	0,672	0,153	orf19.3193	0,917	0,014
orf19.2842	0,856	0,160	orf19.3305	0,644	0,003	orf19.3190	0,425	0,237	orf19.5133	0,682	0,126	orf19.723	0,919	0,054
orf19.1228	0,857	0,043	orf19.5729	0,678	0,195	orf19.6680	0,431	0,207	orf19.4225	0,686	0,125	orf19.5908	0,924	0,038
orf19.3794	0,857	0,101	orf19.1718	0,69	0,057	orf19.7150	0,436	0,215	orf19.4941	0,698	0,150	orf19.3182	0,925	0,024
orf19.4288	0,858	0,056	orf19.6109	0,702	0,072	orf19.4647	0,441	0,222	orf19.5651	0,706	0,141	orf19.6781	0,926	0,085
orf19.3187	0,861	0,174	orf19.454	0,705	0,018	orf19.4318	0,441	0,222	orf19.3308	0,716	0,165	orf19.1275	0,928	0,012
orf19.517	0,865	0,209	orf19.4766	0,706	0,082	orf19.2646	0,452	0,244	orf19.3912	0,723	0,127	orf19.5910	0,931	0,035
orf19.3912	0,879	0,023	orf19.5910	0,707	0,066	orf19.4941	0,459	0,251	orf19.4000	0,723	0,135	orf19.2745	0,937	0,041
orf19.173	0,884	0,206	orf19.1275	0,718	0,130	orf19.4225	0,477	0,243	orf19.5251	0,731	0,172	orf19.4752	0,938	0,047
orf19.4000	0,885	0,054	orf19.6121	0,719	0,055	orf19.2961	0,478	0,227	orf19.3969	0,733	0,101	orf19.4998	0,939	0,075
orf19.5855	0,885	0,025	orf19.921	0,735	0,127	orf19.5133	0,486	0,254	orf19.4145	0,735	0,117	orf19.2612	0,939	0,004
orf19.5343	0,888	0,113	orf19.3188	0,737	0,105	orf19.6781	0,505	0,205	orf19.1496	0,740	0,137	orf19.7247	0,940	0,024
orf19.3753	0,888	0,085	orf19.7436	0,737	0,083	orf19.4752	0,515	0,222	orf19.2842	0,748	0,036	orf19.4869	0,948	0,008
orf19.6985	0,888	0,143	orf19.6817	0,739	0,230	orf19.6109	0,519	0,235	orf19.2745	0,748	0,065	orf19.1623	0,949	0,000
orf19.4251	0,892	0,013	orf19.4318	0,741	0,457	orf19.3193	0,522	0,218	orf19.4853	0,756	0,039	orf19.1718	0,950	0,002
orf19.2961	0,893	0,067	orf19.7570	0,752	0,166	orf19.7401	0,526	0,215	orf19.5548	0,757	0,074	orf19.7371	0,951	0,006
orf19.4450	0,900	0,017	orf19.7401	0,757	0,124	orf19.5910	0,533	0,233	orf19.4166	0,770	0,029	orf19.6985	0,955	0,030
orf19.3625	0,903	0,063	orf19.6124	0,761	0,513	orf19.5251	0,536	0,254	orf19.3876	0,773	0,086	orf19.7436	0,957	0,037
orf19.1275	0,903	0,212	orf19.3182	0,761	0,339	orf19.6798	0,539	0,206	orf19.3928	0,774	0,166	orf19.2753	0,964	0,060

orf19.3928	0,907	0,023	orf19.2730	0,763	0,101	orf19.5729	0,542	0,229	orf19.2119	0,775	0,020	orf19.173	0,965	0,017
orf19.909	0,907	0,132	orf19.2476	0,768	0,167	orf19.5908	0,544	0,231	orf19.1926	0,782	0,119	orf19.4573	0,969	0,019
orf19.2753	0,908	0,066	orf19.5908	0,77	0,317	orf19.3912	0,551	0,238	orf19.3753	0,782	0,077	orf19.3986	0,980	0,006
orf19.6817	0,910	0,154	orf19.2315	0,772	0,095	orf19.5651	0,559	0,260	orf19.3434	0,783	0,032	orf19.1228	0,981	0,105
orf19.4568	0,913	0,020	orf19.4998	0,774	0,052	orf19.7371	0,565	0,220	orf19.391	0,789	0,290	orf19.2747	0,982	0,018
orf19.5249	0,914	0,053	orf19.3986	0,775	0,062	orf19.5001	0,579	0,252	orf19.3809	0,790	0,095	orf19.4767	0,982	0,022
orf19.4573	0,916	0,122	orf19.3187	0,788	0,313	orf19.6985	0,582	0,213	orf19.4251	0,793	0,065	orf19.4766	0,983	0,005
orf19.4662	0,916	0,012	orf19.5343	0,802	0,128	orf19.4853	0,583	0,251	orf19.2356	0,793	0,123	orf19.2476	0,986	0,071
orf19.1623	0,917	0,125	orf19.6680	0,812	0,143	orf19.4670	0,584	0,249	orf19.4568	0,794	0,122	orf19.4318	0,987	0,064
orf19.5908	0,918	0,043	orf19.7247	0,827	0,330	orf19.5326	0,586	0,255	orf19.1973	0,798	0,143	orf19.2808	0,988	0,066
orf19.2646	0,919	0,032	orf19.6514	0,832	0,056	orf19.4766	0,587	0,218	orf19.1035	0,800	0,015	orf19.5917	0,989	0,124
orf19.1168	0,919	0,032	orf19.1623	0,833	0,133	orf19.5097	0,588	0,254	orf19.2808	0,801	0,028	orf19.2842	0,993	0,032
orf19.5651	0,921	0,039	orf19.1499	0,835	0,151	orf19.5343	0,591	0,228	orf19.4524	0,803	0,146	**wt**	**1,000**	**0,179**
orf19.2088	0,921	0,092	orf19.5133	0,839	0,051	orf19.2612	0,600	0,158	orf19.5097	0,804	0,148	orf19.1168	1,002	0,091
orf19.3969	0,925	0,025	orf19.2356	0,846	0,225	orf19.1623	0,604	0,277	orf19.6182	0,807	0,000	orf19.7401	1,003	0,021
orf19.4767	0,926	0,151	orf19.723	0,849	0,027	orf19.6817	0,605	0,207	orf19.4288	0,810	0,057	orf19.3625	1,004	0,103
orf19.3876	0,927	0,001	orf19.4853	0,851	0,016	orf19.4438	0,610	0,245	orf19.4438	0,812	0,120	orf19.5940	1,031	0,073
orf19.2356	0,936	0,063	orf19.1253	0,863	0,106	orf19.7436	0,613	0,215	orf19.2476	0,813	0,104	orf19.3127	1,033	0,039
orf19.4869	0,936	0,076	orf19.7518	0,881	0,205	orf19.7247	0,618	0,212	orf19.5849	0,814	0,069	orf19.2961	1,045	0,117
orf19.5097	0,937	0,006	orf19.6888	0,881	0,133	orf19.4524	0,619	0,247	orf19.4318	0,818	0,020	orf19.6102	1,045	0,110
orf19.4166	0,940	0,014	orf19.7381	0,881	0,325	orf19.3876	0,621	0,237	orf19.7372	0,819	0,101	orf19.3736	1,054	0,007
orf19.5133	0,945	0,083	orf19.4573	0,893	0,165	orf19.1275	0,623	0,270	orf19.4972	0,819	0,049	orf19.5975	1,055	0,090
orf19.971	0,946	0,011	orf19.3736	0,894	0,327	orf19.6888	0,630	0,210	orf19.5001	0,824	0,099	orf19.5924	1,058	0,120
orf19.4318	0,949	0,314	orf19.173	0,9	0,054	orf19.7570	0,630	0,288	orf19.1228	0,824	0,028	orf19.3187	1,061	0,050
orf19.4524	0,952	0,040	orf19.391	0,901	0,194	orf19.4662	0,631	0,248	orf19.3127	0,828	0,029	orf19.7381	1,064	0,062
orf19.5849	0,953	0,060	orf19.2646	0,902	0,210	orf19.3969	0,635	0,239	orf19.1274	0,832	0,078	orf19.4647	1,069	0,071
orf19.1187	0,955	0,074	orf19.2748	0,904	0,248	orf19.5249	0,636	0,226	orf19.723	0,834	0,016	orf19.6182	1,083	0,116
orf19.4647	0,955	0,167	orf19.5651	0,914	0,133	orf19.3753	0,646	0,235	orf19.4056	0,837	0,052	orf19.909	1,089	0,105
orf19.5498	0,956	0,024	orf19.4166	0,921	0,134	orf19.4568	0,649	0,248	orf19.5326	0,838	0,172	orf19.5992	1,097	0,074
orf19.431	0,957	0,086	orf19.1187	0,929	0,168	orf19.723	0,652	0,254	orf19.4545	0,839	0,008	orf19.7570	1,102	0,091
orf19.6109	0,958	0,308	orf19.5249	0,932	0,015	orf19.4450	0,653	0,246	orf19.2753	0,839	0,016	orf19.6874	1,104	0,137
orf19.3736	0,960	0,245	orf19.2647	0,935	0,238	orf19.2315	0,656	0,152	orf19.7570	0,841	0,005	orf19.1973	1,104	0,005
orf19.2647	0,963	0,063	orf19.5548	0,939	0,156	orf19.2088	0,659	0,245	orf19.1168	0,844	0,068	orf19.6038	1,106	0,103
orf19.2315	0,964	0,035	orf19.6102	0,942	0,211	orf19.3928	0,662	0,238	orf19.3625	0,845	0,001	orf19.454	1,110	0,006
orf19.3434	0,965	0,135	orf19.2747	0,942	0,268	orf19.6514	0,662	0,274	orf19.4450	0,845	0,124	orf19.517	1,113	0,118
orf19.2612	0,966	0,132	orf19.4752	0,948	0,393	orf19.7518	0,668	0,286	orf19.5498	0,847	0,055	orf19.3190	1,118	0,087
orf19.4752	0,968	0,029	orf19.4647	0,956	0,381	orf19.3182	0,669	0,158	orf19.6102	0,847	0,018	orf19.3912	1,118	0,114
orf19.6874	0,969	0,135	orf19.4545	0,956	0,181	orf19.4288	0,675	0,244	orf19.5855	0,849	0,076	orf19.7317	1,120	0,134
orf19.4438	0,969	0,097	orf19.4767	0,965	0,155	orf19.3308	0,681	0,234	orf19.971	0,855	0,068	orf19.7319	1,128	0,118
orf19.1253	0,969	0,017	orf19.1274	0,969	0,161	orf19.5338	0,685	0,256	orf19.4670	0,860	0,192	orf19.7374	1,128	0,146
orf19.4972	0,971	0,112	orf19.5001	0,972	0,094	orf19.4145	0,686	0,241	orf19.5380	0,861	0,010	orf19.5001	1,130	0,022
orf19.7372	0,974	0,094	orf19.5992	0,978	0,135	orf19.5975	0,694	0,267	orf19.5338	0,861	0,144	orf19.6514	1,133	0,104
orf19.5026	0,974	0,066	orf19.1973	0,982	0,206	orf19.4000	0,698	0,240	orf19.4752	0,861	0,082	orf19.1035	1,136	0,033
orf19.1069	0,974	0,101	orf19.1496	0,993	0,157	orf19.3736	0,702	0,164	orf19.5026	0,864	0,101	orf19.921	1,137	0,040
orf19.1496	0,978	0,012	orf19.4225	0,996	0,085	orf19.5992	0,704	0,268	orf19.4662	0,865	0,166	orf19.7068	1,140	0,135
orf19.2808	0,979	0,033	orf19.5940	0,999	0,059	orf19.6102	0,705	0,271	orf19.1543	0,868	0,046	orf19.837.1	1,143	0,114
orf19.7317	0,979	0,085	**wt**	**1**	**0,149**	orf19.5940	0,706	0,266	orf19.7359	0,880	0,101	orf19.7583	1,144	0,100
orf19.4225	0,980	0,120	orf19.5097	1	0,008	orf19.5026	0,707	0,253	orf19.4722	0,881	0,007	orf19.166	1,145	0,147
orf19.6781	0,980	0,112	orf19.3308	1,003	0,175	orf19.4251	0,708	0,243	orf19.1032	0,882	0,022	orf19.2119	1,145	0,069
orf19.7247	0,982	0,120	orf19.7359	1,007	0,062	orf19.7359	0,709	0,281	orf19.6121	0,884	0,079	orf19.681	1,149	0,135
orf19.5251	0,983	0,092	orf19.4869	1,007	0,022	orf19.5924	0,711	0,264	orf19.6817	0,886	0,097	orf19.7017	1,149	0,123
orf19.1718	0,984	0,062	orf19.3912	1,014	0,065	orf19.5849	0,713	0,261	orf19.4778	0,890	0,033	orf19.2088	1,152	0,025
orf19.723	0,986	0,172	orf19.3190	1,016	0,114	orf19.4998	0,715	0,223	orf19.6680	0,890	0,100	orf19.6824	1,154	0,150
orf19.6102	0,991	0,166	orf19.5917	1,023	0,067	orf19.3809	0,718	0,236	orf19.5940	0,895	0,018	orf19.391	1,158	0,214
orf19.5975	0,993	0,192	orf19.6182	1,032	0,068	orf19.3986	0,721	0,078	orf19.5908	0,896	0,066	orf19.971	1,159	0,096
orf19.6888	0,993	0,088	orf19.1926	1,033	0,162	orf19.1496	0,727	0,278	orf19.5917	0,897	0,039	orf19.255	1,160	0,148
orf19.7583	0,994	0,093	orf19.2119	1,033	0,188	orf19.5548	0,731	0,259	orf19.5729	0,900	0,098	orf19.4166	1,169	0,047
orf19.5992	0,995	0,097	orf19.1168	1,041	0,035	orf19.173	0,734	0,246	orf19.6781	0,901	0,126	orf19.217	1,170	0,157
orf19.7068	0,997	0,180	orf19.4941	1,043	0,159	orf19.4166	0,739	0,242	orf19.4998	0,903	0,017	orf19.7518	1,171	0,135
wt	**1,000**	**0,109**	orf19.5975	1,044	0,037	orf19.5855	0,743	0,262	orf19.5343	0,907	0,057	orf19.2748	1,178	0,008

orf19.5380	1,003	0,030	orf19.4000	1,048	0,134	orf19.7381	0,749	0,220	orf19.1187	0,912	0,022	orf19.5133	1,187	0,107
orf19.4670	1,003	0,023	orf19.1685	1,056	0,209	orf19.3434	0,749	0,234	orf19.6038	0,915	0,016	orf19.2646	1,191	0,006
orf19.4145	1,004	0,039	orf19.4145	1,057	0,114	orf19.3187	0,755	0,226	orf19.517	0,915	0,045	orf19.431	1,193	0,158
orf19.2748	1,007	0,033	orf19.4438	1,064	0,004	orf19.1187	0,767	0,260	orf19.7583	0,915	0,007	orf19.1032	1,196	0,082
orf19.5326	1,008	0,113	orf19.1543	1,064	0,227	orf19.4778	0,771	0,249	orf19.5910	0,918	0,037	orf19.5548	1,203	0,000
orf19.6824	1,008	0,171	orf19.1035	1,066	0,064	orf19.1973	0,774	0,244	orf19.5249	0,918	0,075	orf19.1253	1,207	0,046
orf19.255	1,008	0,097	orf19.4662	1,068	0,037	orf19.6824	0,778	0,276	orf19.6109	0,919	0,075	orf19.5849	1,209	0,014
orf19.6038	1,009	0,162	orf19.5849	1,07	0,103	orf19.4056	0,779	0,241	orf19.3736	0,927	0,052	orf19.2356	1,212	0,008
orf19.2747	1,010	0,070	orf19.5855	1,07	0,069	orf19.2747	0,782	0,088	orf19.1623	0,928	0,083	orf19.4662	1,215	0,020
orf19.5729	1,013	0,119	orf19.3809	1,071	0,100	orf19.7374	0,796	0,284	orf19.3182	0,930	0,098	orf19.5251	1,216	0,067
orf19.1032	1,017	0,103	orf19.3434	1,074	0,176	orf19.4972	0,799	0,251	orf19.217	0,932	0,006	orf19.3969	1,224	0,025
orf19.217	1,017	0,085	orf19.3753	1,077	0,140	orf19.5380	0,812	0,257	orf19.3187	0,932	0,046	orf19.1926	1,225	0,022
orf19.454	1,019	0,084	orf19.4722	1,078	0,142	orf19.4573	0,816	0,239	orf19.4869	0,933	0,079	orf19.2647	1,236	0,020
orf19.1543	1,020	0,058	orf19.4670	1,081	0,060	orf19.4545	0,823	0,232	orf19.7247	0,934	0,072	orf19.5651	1,237	0,030
orf19.166	1,022	0,098	orf19.4524	1,082	0,059	orf19.7372	0,826	0,283	orf19.6514	0,935	0,092	orf19.3308	1,237	0,012
orf19.5548	1,022	0,033	orf19.1228	1,083	0,059	orf19.5498	0,850	0,258	orf19.2315	0,935	0,099	orf19.5338	1,238	0,096
orf19.5338	1,026	0,109	orf19.255	1,086	0,123	orf19.391	0,852	0,287	orf19.7317	0,938	0,019	orf19.4145	1,245	0,013
orf19.1685	1,028	0,054	orf19.5498	1,091	0,149	orf19.1274	0,854	0,277	orf19.4647	0,938	0,054	orf19.4000	1,246	0,013
orf19.7319	1,032	0,121	orf19.1032	1,093	0,209	orf19.5917	0,856	0,263	orf19.6824	0,939	0,044	orf19.4722	1,247	0,020
orf19.681	1,034	0,090	orf19.7017	1,095	0,227	orf19.7317	0,866	0,279	orf19.5975	0,940	0,026	orf19.4524	1,252	0,025
orf19.7371	1,038	0,187	orf19.681	1,095	0,213	orf19.971	0,867	0,280	orf19.6888	0,940	0,094	orf19.5097	1,257	0,073
orf19.3193	1,040	0,108	orf19.837.1	1,095	0,244	orf19.1926	0,874	0,244	orf19.255	0,941	0,013	orf19.4853	1,258	0,109
orf19.5940	1,041	0,086	orf19.4450	1,096	0,037	orf19.6124	0,876	0,272	orf19.166	0,946	0,058	orf19.5026	1,258	0,074
orf19.1499	1,042	0,019	orf19.7374	1,103	0,274	orf19.7583	0,877	0,289	orf19.2961	0,948	0,014	orf19.1496	1,260	0,015
orf19.1274	1,046	0,008	orf19.971	1,107	0,015	orf19.7068	0,888	0,278	orf19.5924	0,948	0,031	orf19.1274	1,261	0,021
orf19.4722	1,050	0,054	orf19.4288	1,111	0,113	orf19.1035	0,897	0,282	orf19.7436	0,948	0,057	orf19.3434	1,265	0,015
orf19.6121	1,063	0,074	orf19.4056	1,113	0,133	orf19.1543	0,902	0,280	orf19.1275	0,952	0,032	orf19.4450	1,268	0,050
orf19.3190	1,065	0,027	orf19.3928	1,114	0,141	orf19.6182	0,904	0,273	orf19.5992	0,953	0,055	orf19.5855	1,269	0,034
orf19.4766	1,068	0,107	orf19.5380	1,117	0,144	orf19.6038	0,918	0,270	orf19.7518	0,962	0,041	orf19.3809	1,269	0,080
orf19.5924	1,068	0,062	orf19.5326	1,123	0,077	orf19.1032	0,921	0,281	orf19.1718	0,966	0,095	orf19.4778	1,271	0,034
orf19.3182	1,069	0,127	orf19.7372	1,127	0,172	orf19.4767	0,923	0,174	orf19.173	0,966	0,084	orf19.4568	1,272	0,048
orf19.3986	1,071	0,101	orf19.3876	1,129	0,073	orf19.2119	0,944	0,247	orf19.681	0,969	0,037	orf19.3305	1,273	0,111
orf19.391	1,072	0,057	orf19.6824	1,129	0,193	orf19.7017	0,949	0,277	orf19.431	0,970	0,005	orf19.3753	1,274	0,021
orf19.4778	1,073	0,044	orf19.3969	1,133	0,118	orf19.2356	0,958	0,246	orf19.4573	0,971	0,096	orf19.1069	1,278	0,116
orf19.7359	1,074	0,026	orf19.4778	1,135	0,157	orf19.431	0,970	0,288	orf19.7319	0,972	0,040	orf19.5326	1,279	0,082
orf19.6680	1,076	0,103	orf19.5251	1,138	0,180	orf19.1228	0,970	0,277	orf19.837.1	0,977	0,040	orf19.5498	1,279	0,029
orf19.1035	1,079	0,019	orf19.7583	1,14	0,031	orf19.7319	0,974	0,280	orf19.4766	0,980	0,094	orf19.4288	1,280	0,048
orf19.6514	1,083	0,112	orf19.7319	1,145	0,211	orf19.837.1	0,999	0,286	orf19.2747	0,986	0,094	orf19.3928	1,281	0,025
orf19.6182	1,084	0,034	orf19.5026	1,147	0,123	**wt**	**1,000**	**0,152**	orf19.909	0,986	0,031	orf19.4056	1,282	0,069
orf19.7017	1,092	0,115	orf19.5924	1,153	0,066	orf19.6874	1,002	0,277	orf19.7371	0,987	0,042	orf19.1685	1,284	0,018
orf19.837.1	1,096	0,108	orf19.4251	1,157	0,190	orf19.255	1,034	0,287	orf19.2612	0,988	0,052	orf19.7359	1,288	0,027
orf19.5910	1,101	0,056	orf19.4568	1,157	0,132	orf19.4722	1,034	0,250	orf19.6874	0,992	0,074	orf19.4251	1,294	0,039
orf19.7436	1,105	0,066	orf19.517	1,163	0,118	orf19.681	1,038	0,287	orf19.4767	0,993	0,126	orf19.5380	1,298	0,109
orf19.4853	1,108	0,053	orf19.4972	1,17	0,165	orf19.217	1,085	0,288	orf19.7374	0,995	0,049	orf19.3188	1,301	0,084
orf19.7374	1,109	0,073	orf19.7068	1,177	0,025	orf19.4869	1,089	0,179	orf19.7401	0,999	0,105	orf19.1543	1,303	0,049
orf19.7401	1,109	0,107	orf19.5338	1,191	0,144	orf19.517	1,128	0,289	orf19.7017	1,000	0,034	orf19.3876	1,308	0,037
orf19.7570	1,121	0,034	orf19.166	1,191	0,072	orf19.166	1,163	0,290	**wt**	**1,000**	**0,220**	orf19.2730	1,311	0,111
orf19.2730	1,129	0,036	orf19.431	1,192	0,091	orf19.1168	1,218	0,283	orf19.3193	1,014	0,013	orf19.4972	1,321	0,053
orf19.4998	1,153	0,091	orf19.909	1,194	0,138	orf19.2476	1,243	0,244	orf19.6985	1,019	0,029	orf19.4941	1,325	0,041
orf19.7381	1,158	0,172	orf19.7317	1,213	0,184	orf19.909	1,255	0,286	orf19.7068	1,022	0,012	orf19.4438	1,330	0,107
orf19.7518	1,162	0,044	orf19.217	1,244	0,030	orf19.2748	1,285	0,242	orf19.3986	1,080	0,056	orf19.1499	1,334	0,110
orf19.3188	1,171	0,090	orf19.6038	1,257	0,048	orf19.6121	1,387	0,236	orf19.7381	1,095	0,088	orf19.4670	1,338	0,093
orf19.3305	1,185	0,117	orf19.6781	1,309	0,113	orf19.1718	1,583	0,283	orf19.2748	1,258	0,445	orf19.7372	1,339	0,121
orf19.4941	1,187	0,286	orf19.6874	1,438	0,019	orf19.1685	1,773	0,281	orf19.1685	1,284	0,018	orf19.4225	1,370	0,133

RG – Relative Growth; SD – Standard deviation

4.4. Manuscript IV: Gerwien *et al*., *FEMS Microciol Rev* , 2018

Metals in fungal virulence.

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Summary:

Nutritional immunity is an emerging field that has been described as a host defense mechanism against various pathogens. The host has various strategies to withhold or expose essential metals against invading pathogens, which were extensively described in terms of host-bacteria interactions. Recently, the knowledge about nutritional immunity within host-fungi has accumulated and here we compiled the latest information on the current topic. This review provides the outline of iron, zinc, copper, nickel, and manganese homeostasis in fungi. We focused on metal homeostats in pathogenic fungi, including *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and compared it to the extensively studied *Saccharomyces cerevisiae.* The comparison of assimilation, storage, mobilization, and transcriptional regulation mechanisms shed light on how pathogenic fungi cope with metal fluctuations in the host environment.

Own Contribution:

Volha Skrahina wrote the zinc section and revised the review. The other authors designed the concept of the review, generated figures, and wrote the iron, copper, nickel, and manganese sections.

Estimated authors' contributions:

Franziska Gerwien	32**%**
Volha Skrahina	**32%**
Lydia Kasper	2%
Bernhard Hube	4%
Sascha Brunke	30%

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Prof. Bernhard Hube

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REVIEW ARTICLE

Metals in fungal virulence

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One sentence summary: Pathogenic fungi require metals to survive and cause disease in the host. Their complex regulatory, uptake and detoxification systems are often uniquely adapted to conditions *in vivo*. This review compares and contrasts metal homeostasis mechanisms of human fungal pathogens.

Editor: Gerhard Braus

ABSTRACT

Metals are essential for life, and they play a central role in the struggle between infecting microbes and their hosts. In fact, an important aspect of microbial pathogenesis is the 'nutritional immunity', in which metals are actively restricted (or, in an extended definition of the term, locally enriched) by the host to hinder microbial growth and virulence. Consequently, fungi have evolved often complex regulatory networks, uptake and detoxification systems for essential metals such as iron, zinc, copper, nickel and manganese. These systems often differ fundamentally from their bacterial counterparts, but even within the fungal pathogens we can find common and unique solutions to maintain metal homeostasis. Thus, we here compare the common and species-specific mechanisms used for different metals among different fungal species—focusing on important human pathogens such as *Candida albicans*, *Aspergillus fumigatus* or *Cryptococcus neoformans*, but also looking at model fungi such as *Saccharomyces cerevisiae* or *A. nidulans* as well-studied examples for the underlying principles. These direct comparisons of our current knowledge reveal that we have a good understanding how model fungal pathogens take up iron or zinc, but that much is still to learn about other metals and specific adaptations of individual species—not the least to exploit this knowledge for new antifungal strategies.

Keywords: transition metals; pathogenic fungi; nutritional immunity; metal homeostasis; host–pathogen interactions; regulatory networks

INTRODUCTION

Fungi are frequently underestimated as causes of disease and death worldwide—by the public, by health practitioners, and even by national and global health organizations (Brown *et al.* 2012). Because of their often high mortality rates, infections with invasive fungi from genera as diverse as *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Paracoccidioides* or *Blastomyces* are responsible for about one and a half million deaths per year (Brown *et al.* 2012), and non-fatal infections will affect most people at least once in their lifetime, with correspondingly high costs for healthcare systems worldwide. The search for fungal virulence factors and thus potential new drug targets in these eukaryotic pathogens is therefore all the more important.

Metals play a surprisingly central role in infection processes, as they serve as cofactors in a multitude of enzymes—including many with direct and indirect roles in virulence, such as metal-dependent superoxide dismutases (SODs), metalloproteases or melanin-producing laccases. Especially the first-row transition metals—manganese (Mn), iron (Fe), cobalt (Co), nickel

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(Ni) and copper (Cu)—provide the necessary redox and catalytic activity for many important biological processes. Their ionization energies increase slowly both over the row and for subsequent ionization events in the same metal. In the case of first-row transition metals, this is due to the shielding effect of their 3d-electrons on the 4s-electrons, and these are first lost during ionization. In fact, all these transition metals thus have a stable +2 oxidation state (lacking the 4s-electrons) and generally many additional stable states (up to seven in the case of Mn), which allows them to readily change their oxidation states in biological reactions. Zinc (Zn), with its single oxidation state (+2) and its filled d-orbital, is a notable exception, but nonetheless plays important roles especially in eukaryotic gene regulation.

The host is similarly dependent on metals, and should theoretically present a near optimal, metal-rich environment for infecting microbes. However, this is counterintuitively not the case, a fact that helps our intact immune system to fend off pathogenic fungi and bacteria. This is due to a process aptly named 'nutritional immunity', where the host actively sabotages and counteracts metal uptake by microorganisms (Weinberg 1975) and to make matters worse—as seen from the pathogen's side—can also fight invaders by deploying toxic levels of certain metals (Hood and Skaar 2012). Iron, copper and manganese, for example, are intrinsically toxic via Fenton chemistry (Fenton 1894), the metal-catalyzed generation of oxygen radical species from hydrogen peroxide, which at high metal concentrations results in oxidative damage to the microbes (Higson, Kohen and Chevion 1988; Touati 2000). Furthermore, many of the common biological metals have similar divalent cation properties in binding ligands, but strikingly different catalytic functions. Mismetallation, i.e. the replacement of an enzyme's metal cofactor by a different metal by host-induced metal excess and oxidative stress (reviewed in Imlay 2014), could thus inhibit the function of microbial enzymes that require defined metals as cofactors (Macomber and Imlay 2009; McDevitt *et al.* 2011; Veyrier *et al.* 2011). Consequently, the pathogens must keep these essential metals within strict homeostatic boundaries even when moving through rapidly changing metal microenvironments within the host. Finally, in biologically relevant pH ranges, these metals are frequently more soluble under acidic conditions, which results in often pH-dependent systems of metal homeostasis, many of which are described below.

Many of the metal conditions in microbial organisms still reflect the environment that we envision to have existed during the emergence of life. Then, iron was mainly present in its ferrous form (Fe^{2+})—due to the anoxic environment, which also led to copper and other soft metals to be trapped away in sulfide minerals. Especially eukaryotes, like fungi, later learned to include zinc and, to a certain extent, copper into the spectrum of biologically useful metals. Still, the profound differences between the evolutionary inherited patterns of metal use and the modern lower availability of iron (mostly ferric (Fe^{3+}) rather than ferrous, due to the newly oxic conditions), and the relative abundance of soft metals, like copper, presents a continuing challenge to microbes, which nonetheless may have 'trained' the microorganisms to better deal with the metal-based nutritional immunity of mammals.

In fact, pathogenic fungi have developed often complex and advanced detection and signaling networks to upregulate the import of specific metals in times of need. Frequently, biological processes that rely on these metals are downregulated by dedicated regulators, reducing the consumption and liberating the bound metal. Under metal excess, often (but not always) a different regulator stops the expression of importers and initiates the sequestration of surplus metal to special proteins like metallothioneins (MTs) or to the vacuole, which serves as an overflow basin and emergency reservoir for many different metals. Many transporters have evolved that allow the transport of the charged metal ions over the plasma or vacuolar membranes, but unspecific transport of several metals by the same transporter is not uncommon—bringing with it the danger of the loss of full control over the metals that enter the cell and possibly leaving the microbe vulnerable to metal toxicity (Liu *et al.* 1997; Li and Kaplan 1998; Viau *et al.* 2012; Caetano *et al.* 2015).

Excellent recent reviews exist on many aspects of bacterial metal use, and among those we highly recommend (Palmer and Skaar 2016) for readers interested in non-fungal systems. On the topic of nutritional immunity, we recommend (Hood and Skaar 2012) for an outstanding overview of metal-related bacteria–host interactions, and (Crawford and Wilson 2015) for a view on common fungal pathogens. For an in-depth view on individual metals and their role in microbial pathogenesis, we refer the reader to Garcia-Santamarina and Thiele (2015) for copper, and for iron to Ganz and Nemeth (2015) and Soares and Weiss (2015) for a host view and Bairwa, Hee Jung and Kronstad (2017) for the fungal side.

In this review, we compile and compare strategies that fungi employ to obtain metals during pathogenesis, and we provide examples for different homeostatic mechanisms, and how they connect to fungal virulence. To this end, we summarize here the basic principles of homeostatic regulation in pathogenic fungi for iron, zinc, copper and manganese—metals for which a sufficiently large body of literature exists. The direct comparisons of known mechanisms among fungi will, we hope, allow the reader to discover common principles and identify open questions in order to complete our picture of the role of metals in fungal infections.

IRON

Most texts on microbial metal homeostasis start with a focus on iron. This is for good reason, as iron is the most abundant of the trace metals in organisms and arguably the one with the most diverse roles in cellular processes. These include central metabolic pathways such as oxygen transport, the tricarboxylic acid (TCA) cycle or electron transport chains, mostly via incorporation of iron or the iron-containing prosthetic group heme into the active centers of key enzymes. For these reasons, iron is an essential metal in nearly all organisms (*Borrelia burgdorferi*, the causative agent of Lyme disease, is one of the rare and notable exceptions; Posey and Gherardini 2000). While the ubiquity of iron is related to its chemical redox properties, namely the capacity to readily switch between the ferric and the ferrous form, this same quality is also at the root of the problems that can be caused by iron in many biological systems. For instance Fe^{3+}, the prevalent form under aerobic conditions, is essentially insoluble in water and hence inaccessible to most microbes. Fe^{2+} in contrast is much more soluble, but at the same time more prone to elicit iron-induced toxicity mediated by the formation of radicals via the Fenton reaction. Additionally, iron, similar to copper, has a high affinity to replace other metals in enzymatic reactive centers, a mismetallation that usually results in a disruption of the enzymatic function (Vance and Miller 1998; Martin and Imlay 2011).

Accordingly, vertebrates and microorganisms alike have developed sophisticated strategies to ensure solubility, distribution and steady supply of iron while keeping its homeostatic

levels sufficiently low to prevent toxicity. In vertebrates, this includes the almost complete binding of iron via a plethora of transport and storage proteins, such as hemoglobin, transferrin, lactoferrin and ferritin (reviewed in Wang and Pantopoulos 2011). During infection, microbial access to iron (and other metals) is actively restricted even further by nutritional immunity mechanisms (Weinberg 1975). This occurs at the systemic level by hepcidin-induced reduction of circulating iron (Nemeth et al. 2004) and at the tissue level by the active redistribution of iron away from sites of infection (Potrykus et al. 2013). In these processes, iron is shuttled to intracellular stores to keep it out of reach of invading pathogens—predominantly in macrophages, which also act as natural heme recycling sites via phagocytosis of senescent erythrocytes (reviewed in Wang and Pantopoulos 2011).

However, a range of microbial pathogens have adopted an intracellular lifestyle and use macrophages as hiding places from the immune system, or even as a source of nutrients and metals for their own growth. This includes many pathogenic fungi such as the dimorphic ascomycete *Histoplasma capsulatum* (Newman et al. 1994; Hwang et al. 2008), the basidiomycete *Cryptococcus neoformans* (Levitz et al. 1997), the yeast-like ascomycete *Candida glabrata* (Nevitt and Thiele 2011; Seider et al. 2014) and other dimorphic ascomycetes e.g. *Paracoccidioides brasiliensis* (Cano et al. 1994) or *Blastomyces dermatitidis* (Sterkel et al. 2015). All these species are able to survive phagocytosis and replicate inside macrophages, and they use diverse strategies in order to exploit the intracellular iron stores of macrophages, not all of which have yet been elucidated (Hilty, Smulian and Newman 2008, 2011; Nevitt and Thiele 2011; Hu et al. 2015).

Iron homeostasis and uptake

Pathogens have evolved elaborate systems to acquire iron from their environment (Fig. 1). A common theme in iron uptake is the utilization of siderophores, a heterogeneous class of small molecules, which are secreted by bacteria and fungi to bind extracellular ferric iron with extremely high affinity. This is achieved by coordinating Fe^{3+} by normally six oxygen ligands per molecule in an octahedral geometry, although siderophores with less donor atoms per molecule can bind in stochiometries different from 1:1 or use water as an additional oxygen donor. Siderophore–iron complexes are then either taken up directly or they deliver their precious load to receptors of the microbe's surface for uptake via specific transporters (reviewed for fungi in Haas, Eisendle and Turgeon 2008). Like in bacteria, many different classes of fungal siderophores are known, such as the most commonly produced hydroxamates [triacetylfusarinine C (Charlang et al. 1981; Oide et al. 2006; Schrettl et al. 2007), coprogens (Matzanke et al. 1987), ferrichromes (Neilands 1952), rhodotorulic acid (Muller, Barclay and Raymond 1985)], polycarboxylates produced by zygomycetes (Thieken and Winkelmann 1992) and phenolates-catecholates, which are present in wood-rotting fungi (Fekete, Chandhoke and Jellison 1989). Some fungal siderophores have highly specialized roles: *Aspergillus fumigatus* and *A. nidulans* ferricrocins, for example, are found inside the fungus rather than being secreted, and are involved in intracellular iron homeostasis and storage (Eisendle et al. 2006; Schrettl et al. 2007; Gsaller et al. 2012). Similarly, ferrichromes of the plant-pathogenic fungi *Ustilago sphaerogena* and *U. maydis* can be secreted or store iron intracellularly (Ecker, Lancaster and Emery 1982; Budde and Leong 1989). Importantly, Fe^{3+} bound to siderophores, due to their strongly negative redox potential, is not readily reduced to Fe^{2+} and hence will not generate hydroxyl radicals (Cornish and Page 1998). By this mechanism, intracellular siderophores can help to protect microbes from the toxic effects of iron (Eisendle et al. 2006).

Overall, siderophore producers are widespread in the fungal kingdom and include animal and human pathogens such as *Aspergillus* spp. (Zähner et al. 1963; Nilius and Farmer 1990; Gressler et al. 2015), *H. capsulatum* (Howard et al. 2000), *Rhodotorula pilimanae* (Carrano and Raymond 1978), *Neurospora crassa* (Horowitz et al. 1976), *Paracoccidioides* spp. (Silva-Bailao et al. 2014) and the plant pathogens *U. maydis* (Budde and Leong 1989) and *Alternaria brassicicola* (Oide et al. 2006), among many others. In fact, siderophores are essential for the virulence of most fungal pathogens producing them. Deletion mutants lacking siderophore synthesis genes show severe virulence defects in *A. fumigatus* (Schrettl et al. 2004; Hissen et al. 2005), and also in *H. capsulatum* (Hwang et al. 2008). Consequently, the host has been shown to sequester fungal (and bacterial) siderophores via siderocalins, special siderophore-binding lipocalins (Goetz et al. 2002; Leal et al. 2013). Notably, the cellular energy cost to sustain siderophore synthesis is rather high for the microbe. Hence, biosynthesis is generally tightly controlled and activated solely upon significant iron shortage (Mei, Budde and Leong 1993; Oberegger et al. 2001). In addition, many fungal species, including *C. albicans*, *C. glabrata* or *Saccharomyces cerevisiae*, as well as *Cr. neoformans*, *Geotrichum candidum* and *Rhizopus* spp., lack the key enzyme L-ornithine N5-oxygenase (Sid1/SidA), which is needed for the initiation of hydroxamate siderophore biosynthesis, and they thus do not produce their own siderophores (reviewed in Haas, Eisendle and Turgeon 2008). Controversially, siderophore production was reported for *C. albicans* (Ismail, Bedell and Lupan 1985), but no putative biosynthesis genes were subsequently found in the genome.

Lacking their own biosynthetic machinery, these species often rely on xenosiderophores, i.e. siderophores produced by other fungi or bacteria. Dedicated xenosiderophore transporters with different substrate specificities have evolved, e.g. Sit1 homologs for hydroxamate-type fungal siderophores in *C. glabrata* (Nevitt and Thiele 2011), *C. albicans* (Heymann et al. 2002; Lesuisse et al. 2002), *Cr. neoformans* (Tangen et al. 2007) and *S. cerevisiae* [Arn1–4, with Arn3 and Arn4 specific for bacterial ferroxamines and Enterobactin B, respectively (Heymann, Ernst and Winkelmann 2000a,b; Yun et al. 2000)] and in many other fungi. *Candida glabrata* Sit1 enhances fungal survival in macrophages (Nevitt and Thiele 2011), and *C. albicans* Sit1 is required for invasion of human epithelial cells *in vitro* (Heymann et al. 2002); in the absence of xenosiderophores, these observations seem puzzling, and although mammals were recently found to produce siderophores (Devireddy et al. 2010), these are similar to enterobactin und thus unlikely to be taken up via Sit1. Accordingly, *SIT1* deletion causes no attenuation in virulence of *C. albicans* in a systemic mouse model of infection (Hu et al. 2002). Similarly, *Cr. neoformans* Sit1 deletion mutants showed changes in melanin and capsule formation and in cell wall density, but were not reduced in virulence (Tangen et al. 2007)—however, there are six more potential siderophore transporters encoded in the *Cr. neoformans* genome (Jung and Kronstad 2008).

Overall, the ability to use a broad spectrum of xenosiderophores likely reflects microbial competition for iron. This would make such a strategy advantageous when close interspecies contacts are frequent, such as in biofilms in the oral cavity, gut or vagina, as well as generally in co-infections. However, in the absence of any evident producer, the role of xenosiderophore binding during dissemination in blood or host tissue remains unclear at best. In these environments,

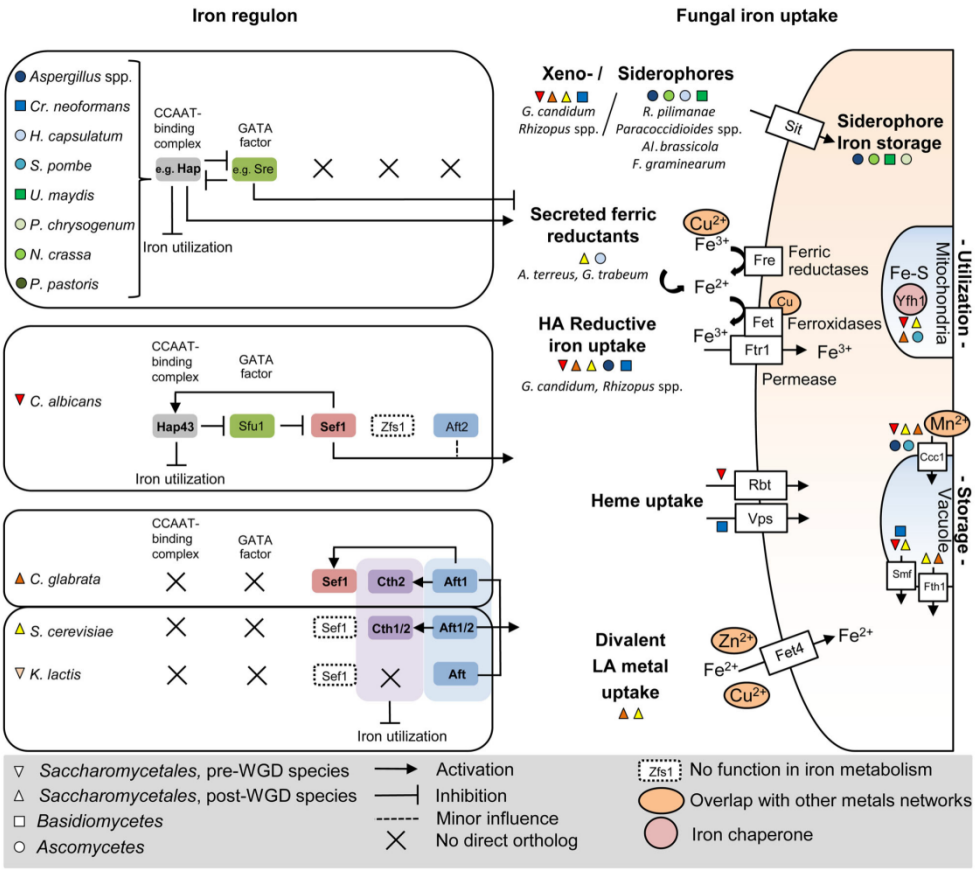

Figure 1. Fungal iron homeostasis. Regulation of iron homeostasis (left panel side) is shown for different fungal species (species is color coded, shape defines phylogenetic ancestry according to Gabaldon *et al.* 2013). Major transcription factors upregulated during iron starvation to initiate fungal iron uptake (right panel side) are written in bold. Functional orthologs are color shaded and aligned vertically, X indicates lack of ortholog and a white box with dashed borders indicates that an ortholog is present but not involved in iron homeostasis. HA, high affinity; LA, low affinity.

it seems more important that many fungi have developed multiple mechanisms to directly exploit iron-binding molecules of the host. *Candida albicans* shows an impressive versatility in using host sources and can directly or indirectly obtain iron from hemoglobin (Moors *et al.* 1992), hemin (Santos *et al.* 2003), ferritin (Almeida *et al.* 2008) and transferrin (Knight *et al.* 2005). Similarly, *Cr. neoformans* can use transferrin (Jung *et al.* 2008), heme and hemin (Jung *et al.* 2008; Cadieux *et al.* 2013; Hu *et al.* 2015), and *H. capsulatum* is known to obtain iron from transferrin and hemin (Timmerman and Woods 1999; Foster 2002), but *Aspergillus* spp. appear to be unable to acquire iron from heme (Vaknin *et al.* 2014).

In hemoglobin, iron is incorporated in heme in its ferrous form and can be acquired by *C. albicans* and *Cr. neoformans* with specific heme uptake mechanisms. The former relies on a family of heme receptors [Rbt51 (Moors *et al.* 1992; Weissman and Kornitzer 2004)] and hemophores [Rbt5, Pga7, Csa2 (Weissman and Kornitzer 2004; Weissman *et al.* 2008; Kuznets *et al.* 2014; Nasser *et al.* 2016)] for initial uptake followed by ESCRT complex-mediated internalization into the vacuole via the endocytic pathway (Weissman *et al.* 2008). In *Cr. neoformans*, the ESCRT complex similarly has a pronounced role in heme utilization [Vps23, Vps22, Snf7 (Hu *et al.* 2013, 2015)] along with the putative hemophore Cig1 (Cadieux *et al.* 2013). The internalized heme-bound iron is then released by a heme oxygenase, which has been described in many *Candida* species and in *S. cerevisiae* to recycle self-generated heme (Santos *et al.* 2003; Kim *et al.* 2006). Other host iron sources containing Fe^{3+} can also be taken up directly, or, more commonly, the bound Fe^{3+} is first extracted from host molecules (or siderophores) on the cell surface via ferric reductases. Fe^{2+} is then oxidized again by permease-coupled multicopper ferroxidases followed by transmembrane transport of Fe^{3+} via high-affinity permeases to complete the uptake process. This system is especially important

for virulence in non-siderophore producing fungi such as *Cr. neoformans* (Jung *et al.* 2009; Han, Do and Jung 2012), *C. albicans* (Ramanan and Wang 2000; Fang and Wang 2002; Knight *et al.* 2005; Cheng *et al.* 2013) and *C. glabrata* (Srivastava, Suneetha and Kaur 2014), which heavily rely on the reductive pathway for iron uptake to facilitate growth and virulence (Srivastava, Suneetha and Kaur 2014; Gerwien *et al.* 2016, 2017). In contrast, while *A. fumigatus* siderophore synthesis mutants were dramatically attenuated in virulence (Hissen *et al.* 2005), defects in reductive iron assimilation had no significant effect (Schrettl *et al.* 2004). Similarly, other siderophore producers, such as *Fusarium graminearum* (Greenshields *et al.* 2007) or *Al. brassicicola* (Oide *et al.* 2006), cannot fully compensate the loss of siderophore-mediated iron uptake by the reductive uptake system alone.

As described above, the reductive uptake system comprises reductase and linked permease/ferroxidase functions. Pathogenic fungi commonly have large families of cell-surface NAD(P)H-dependent ferric reductases at their disposal, such as *Cr. neoformans* (eight known reductases) (Saikia *et al.* 2014), *C. albicans* (18 putative) (Jeeves *et al.* 2011; Xu *et al.* 2014b) or *A. fumigatus* (15 putative) (Blatzer, Binder and Haas 2011)—with no number currently available for *H. capsulatum*. *Candida albicans* Fre2, Fre5/Frp1 and Fre9 (Bensen *et al.* 2004; Baek, Li and Davis 2008) are expressed under alkaline conditions, and there are indications that Fre2 might be secreted or shedded under azole treatment (Sorgo *et al.* 2011). In *Cr. neoformans*, transcription levels of Fre3 seem to be associated with virulence: RNAi suppression of *FRE3* decreased survival in macrophages, while artificial upregulation led to increased virulence in mice (Hu *et al.* 2014).

Ferric reductases are best characterized in *S. cerevisiae*, where, despite obvious redundancy, the nine known members each play specific roles in siderophore-Fe reduction (Fre1, Fre2, Fre3, Fre4) (Martins *et al.* 1998; Yun *et al.* 2001), copper reduction (Fre1, Fre2, Fre7) (Martins *et al.* 1998) and presumably in intracellular transmembrane shuttling at the vacuole (Fre6) (Huh *et al.* 2003). In *C. albicans*, similar specific functions have been attributed to Fre7 and Fre10 as cupric reductases (Jeeves *et al.* 2011). *Candida glabrata* (Srivastava, Suneetha and Kaur 2014) and the fission yeast *Schizosaccharomyces pombe* (Roman *et al.* 1993) are notable exceptions, since they each possess only two ferric reductase genes. In *C. glabrata*, the lack of *FRE6* has been associated with attenuated virulence in a *Drosophila* model (Brunke *et al.* 2015) and slightly decreased kidney fungal burdens in mice (Srivastava, Suneetha and Kaur 2014). However, our own work has shown that both Fre6 and Fre8 might have roles other than ferric or cupric reduction in *C. glabrata*, since this fungus does not exhibit evident surface ferric reductase activity (Gerwien *et al.* 2017). Finally, low-affinity broad-spectrum metal transporters for iron, copper and zinc have been identified in *S. cerevisiae* (Fet4) (Dix *et al.* 1994) and in *C. glabrata* (Fet4) (Srivastava, Suneetha and Kaur 2014; Gerwien *et al.* 2016) with possible orthologs in *Cr. neoformans* (Jacobson, Goodner and Nyhus 1998; Jung *et al.* 2008) and in *Sc. pombe* (Dainty *et al.* 2008).

Non-siderophore secreted molecules with the capacity to bind and reduce iron are also known in fungi. For instance, *H. capsulatum* uses the glutathione-dependent γ-glutamyltransferase Ggt1 to extracellularly reduce ferric iron from siderophores, transferrin and hemin (Timmerman and Woods 1999; Timmerman and Woods 2001; Zarnowski *et al.* 2008). Non-enzymatic ferric reductants are also excreted by this fungus (Timmerman and Woods 1999), although their exact nature is still unknown. In *Cr. neoformans*, 3-hydroxyanthranilate has been identified as an extracellular ferric reductant, but additional active compounds seem to exist (Nyhus, Wilborn and Jacobson 1997; Jacobson, Goodner and Nyhus 1998; Jung *et al.* 2008). As melanized *Cr. neoformans* cells reduce iron at a much higher rate than non-melanized cells, ferric reduction activity may be associated with this polymer (Nyhus, Wilborn and Jacobson 1997). In *S. cerevisiae*, excretion of anthranilate and 3-hydroxyanthranilate correlates with ferric reduction capacity in the extracellular medium, although, counterintuitively, cells grown in iron-rich medium show a higher secretion than those in iron-poor medium (Lesuisse *et al.* 1992). Likewise, culture supernatants of *C. albicans*, *C. glabrata* and *S. cerevisiae* show ferric reduction activity, which depends on a so far unknown low-molecular-weight compound (Gerwien *et al.* 2017), and *A. terreus* has recently been shown to secrete terrein under iron starvation, which acts as a ferric reductant and can partially rescue strains defective in siderophore biosynthesis (Gressler *et al.* 2015).

In a similar fashion, the active lowering of the environmental pH can increase iron bioavailability, either by increasing the overall solubility or via pH-dependent release of iron from host molecules such as transferrin (Lestas 1976). *Histoplasma capsulatum* is known to exploit this strategy inside macrophages, keeping the intraphagosomal pH at 6.5 (Eissenberg, Goldman and Schlesinger 1993). This is alkaline enough to inhibit phagolysosome function, but acidic enough to keep iron accessible and possibly even release it from host transferrin (Newman *et al.* 1994; Hilty, Smulian and Newman 2008). In fact, this strategy was found to be essential for intracellular growth and virulence of *H. capsulatum* (Hilty, Smulian and Newman 2008). Similar mechanisms are probably also used by other fungi with the ability to manipulate phagosomal pH, like, for example, *C. glabrata* (Kasper *et al.* 2014).

Excess iron is stored both as a stockpile for times of need and to avoid its toxicity at high concentrations. Storage is mediated either by vacuolar polyphosphates or by intracellular siderophores (see above); with the exception of zygomycetes, ferritin-like molecules with this purpose are so far unknown in fungi (Carrano, Bohnke and Matzanke 1996). In *S. cerevisiae*, the transporter Ccc1 mediates vacuolar iron (and manganese) import (Lapinskas, Lin and Culotta 1996; Li *et al.* 2001), while export is controlled by Smf3 (Portnoy, Liu and Culotta 2000) or a complex consisting of Fth1/Fet5 coupled to a ferric reductase, resembling the reductive uptake system of the plasma membrane (Urbanowski and Piper 1999). Ccc1 orthologs with similar roles in iron storage exist in *C. glabrata* (Gerwien *et al.* 2016), *C. albicans* (Xu *et al.* 2014a), *A. fumigatus* (Gsaller *et al.* 2012), *A. nidulans* (Eisendle *et al.* 2006) and *Sc. pombe* (Mercier, Pelletier and Labbe 2006), indicating that vacuolar iron storage is important in both siderophore producers and non-producers. Similarly, Smf3 has been associated with intracellular iron homeostasis in *S. cerevisiae* (Portnoy, Jensen and Culotta 2002) and in *C. albicans* (Xu *et al.* 2014a), and an ortholog is present in *C. glabrata*. Deletion of *C. glabrata* Fth1 or Fet5 does not cause sensitivity to the iron chelator bathophenanthroline disulfonate (Srivastava, Suneetha and Kaur 2014), although *FTH1* was found to be iron regulated (Gerwien *et al.* 2016). *Aspergillus nidulans* and *Sc. pombe* finally lack orthologs for both genes—however, in the latter, Abc3 has been suggested to have a similar role in vacuolar iron mobilization (Pouliot *et al.* 2010).

The organelles with the highest need for iron are mitochondria. Here iron-sulfur (Fe-S) clusters are synthesized as prosthetic group for respiratory chain complexes, the TCA cycle and various other metabolic processes. Consequently, a highly conserved short-term storage molecule has evolved in fungi and mammals: the mitochondrial matrix iron chaperone Yfh1 (Huynen *et al.* 2001), which has been found in *S. cerevisiae* (Babcock

et al. 1997; Wilson and Roof 1997), *Sc. pombe* (Fxn1) (Wang *et al.* 2014), *C. albicans* (Santos *et al.* 2004) and *C. glabrata* (Srivastava, Suneetha and Kaur 2014).

Iron-sensing and transcriptional regulation

Regulation of fungal iron homeostasis has mostly been studied in the model yeast *S. cerevisiae*. However, baker's yeast is barely representative of other fungi, since it employs a rather unusual regulation system, which among the pathogenic fungi has so far only been found in the closely related *C. glabrata* (Gerwien *et al.* 2016). In both species, an Aft transcription activator (Aft1 and Aft2 in *S. cerevisiae*) upregulates genes involved in Fe uptake under iron limitation (Yamaguchi-Iwai, Dancis and Klausner 1995; Ueta *et al.* 2012). Mechanistically, this is mediated by Fe-S clusters produced in the mitochondria, which—when present—bind the glutaredoxins Grx3 and Grx4 and enable them to interact with Aft1 to remove it from its promoter targets (Rutherford *et al.* 2005; Ueta *et al.* 2012). Such Fe-S clusters also play a role in adaptation to high iron, as they can activate the high iron-responsive regulator Yap5 (Li *et al.* 2012). Its limited range of target genes includes *CCC1* (Li *et al.* 2008), coding for the vacuolar iron importer (Li *et al.* 2001), and *CUP1* (Pimentel *et al.* 2012), encoding a copper-binding MT.

During Fe starvation, iron-requiring processes are post-transcriptionally further downregulated via degradation of mRNAs that carry the target sequence 5′-(U)UAUUUAU(U)-3′ in their 3′UTR region. This process is mediated by the combined action of the RNA-binding proteins Cth1 and Cth2 in *S. cerevisiae* (Shakoury-Elizeh *et al.* 2004; Puig, Askeland and Thiele 2005; Puig, Vergara and Thiele 2008; Martinez-Pastor *et al.* 2013) and by a single Cth2 ortholog in *C. glabrata* (Gerwien *et al.* 2016). Thus, *C. glabrata* and *S. cerevisiae* (and likely their closest relatives) uniquely share the Aft/Cth iron regulatory system, although their opportunistic pathogenic and environmental lifestyles would at first glance suggest the need for vastly different iron homeostasis mechanisms. Interestingly, further Aft orthologs with roles in iron homeostasis have been identified in *Kluyveromyces lactis* (Conde e Silva *et al.* 2009), also a part of the *Saccharomycetaceae* clade (Gabaldon *et al.* 2013), and surprisingly in the evolutionary more distant yeast *C. albicans* (Liang *et al.* 2010; Xu *et al.* 2013). However, *K. lactis* lacks any Cth2 ortholog, whereas the one present in *C. albicans* (Zfs1) has no function in iron homeostasis, but influences biofilm formation (Wells *et al.* 2015). Notably also, *C. albicans* Aft2 has only a very minor function in iron homeostasis regulation (Liang *et al.* 2010; Xu *et al.* 2013), since, like most other fungi, *C. albicans* relies on a different iron regulation strategy.

This other system has so far been found (often with slight variations) in *C. albicans*, *Cr. neoformans*, both *A. fumigatus* and *A. nidulans*, and *Sc. pombe*. It usually comprises two repressors: a GATA transcription factor for the downregulation of iron acquisition (called Sfu1, Cir1, SreA, or Fep1 in these fungi) and a CCAAT-binding complex to downregulate iron consumption pathways (Hap43, HapX, HapX, or Php4) (Haas *et al.* 1999; Oberegger *et al.* 2001; Tuncher *et al.* 2005; Mercier, Pelletier and Labbe 2006; Hortschansky *et al.* 2007; Schrettl *et al.* 2008; Jung *et al.* 2010; Schrettl *et al.* 2010; Chen *et al.* 2011; Hsu, Yang and Lan 2011; Kronstad, Hu and Jung 2013). In *H. capsulatum* (Hwang *et al.* 2012), *N. crassa* (Zhou, Haas and Marzluf 1998), *Penicillium chrysogenum* (Haas, Angermayr and Stoffler 1997) and *U. maydis* (Voisard *et al.* 1993), a GATA factor (Sre1, Sre, SreP, Urbs1) with an iron-regulatory function has been characterized, but in these fungi, a complete iron-related CCAAT-binding complex has not been described yet. It is likely to be present, though, as both components play complementary roles for the efficient adaption to varying iron levels: under iron depletion, which is frequently encountered during active infections, the CCAAT-binding complex represses the iron-consuming cellular processes. At the same time, it indirectly induces iron acquisition by repressing the GATA transcription factor to alleviate its repressive effect on iron uptake. The latter function of the GATA transcription factor is in turn important under iron-replete conditions likely encountered by *C. albicans* cells commensally growing in the mammalian gut (Chen *et al.* 2011). In these environments, it also downregulates the CCAAT-binding complex, increasing the iron-consuming cellular processes. In *A. fumigatus*, HapX was recently shown to be important under both iron starvation and excess. Through different domains, this factor can either repress consumption or activate vacuolar sequestration of iron, depending on the current concentration of the metal (Gsaller *et al.* 2014). With these central roles, it is not surprising that a deletion of the CCAAT-binding complex results in attenuated virulence in *Cr. neoformans* (Jung *et al.* 2010), *C. albicans* (Hsu, Yang and Lan 2011; Singh *et al.* 2011) and *A. fumigatus* (Schrettl *et al.* 2010). Deletion of the GATA transcription factor leads to more varied outcomes, from complete avirulence in *Cr. neoformans* (Jung *et al.* 2006) to unchanged, wild-type level virulence in mouse infections for *A. fumigatus* Δ*sreA* (Schrettl *et al.* 2008) and *C. albicans sfu1*Δ/Δ (Chen *et al.* 2011). Notably, however, the *C. albicans sfu1*Δ/Δ mutant is severely defective in GI tract colonization, where iron is abundant (Chen *et al.* 2011).

Candida albicans adds a twist to this established system, as this fungus has incorporated a third regulator into the GATA/CCAAT partnership. Sef1 is an activator of Hap43 expression (Chen *et al.* 2011) and is required for full virulence (Chen and Noble 2012). Possibly, the two lifestyles of *C. albicans*—both as a pathogen and as a commensal in the gut where iron levels can change rapidly through food intake and microbial competition—require an additional stabilizing element in iron homeostasis regulation (Chen *et al.* 2011). Interestingly, a Sef1 ortholog is also present in *C. glabrata*, like *C. albicans* a commensal of mucosal surfaces, but with a vastly different regulatory network, and this has been shown to play an (albeit less pronounced) role in iron homeostasis (Gerwien *et al.* 2016).

Finally, with the close connection between pH and metal solubility, some fungi, such as *C. albicans* and *Cr. neoformans*, use the pH-responsive factor Rim101 to detect alkaline pH as a marker for iron starvation and signal to upregulate the iron acquisition systems (Bensen *et al.* 2004). Consequently, a *C. albicans rim101*Δ/Δ mutant is attenuated in virulence (Davis *et al.* 2000). Similarly, a *Cr. neoformans rim101*Δ/Δ mutant is unable to utilize heme (Cadieux *et al.* 2013), but was found to be hypervirulent (O'Meara *et al.* 2010) likely because of an (unrelated) defective shedding of capsule polysaccharides, which results in a hyperactivation of the host immune response (O'Meara *et al.* 2010, 2013).

The evolution of these various and partly redundant systems for iron homeostasis throughout fungal pathogens reflects the importance of this particular micronutrient. Adaptations occurred in response to host-induced scarcity, to conditions of varying pH, and to the changing availability of host iron sources. Our current knowledge on these adaptations is already being used to develop new therapeutic approaches, for example, by supporting the host in its iron restriction during fungal infections (reviewed in Bruhn and Spellberg 2015; Lamb 2015) or by using fungal iron acquisition systems as targets for potential vaccines—as has been done with *C. albicans* Als3, although its involvement in iron uptake was not known at that time (Spellberg

et al. 2006, 2008). It is therefore noteworthy that, beyond well-researched examples such as *C. albicans* or *A. fumigatus*, many fungal iron acquisition strategies are likely still unknown to us.

ZINC

Zinc is a structural and catalytic co-factor for many proteins, including the ubiquitous zinc finger DNA-binding proteins. Recently, zinc was also shown to be an intracellular second messenger in various transduction signaling pathways (Yamasaki *et al.* 2007). In fact, zinc is the second most abundant trace metal in the human body: there are more than 300 zinc-dependent enzymes, and ≈10% of human genes code for zinc-binding proteins (Andreini *et al.* 2006a). The importance of zinc is sadly evident in the two billion people who suffer from zinc deficiency, especially in developing countries: A lack of zinc leads to thymic atrophy and lymphopenia, and weakens both the innate and adaptive immune responses: phagocytosis, cytokine production by macrophages, host defense by neutrophils and natural killer cells, and antibody secretion of both T and B cells are all impaired under zinc deficiency (reviewed in Prasad 2012).

Like for humans, zinc is of high importance for microorganisms. Within the fungi, zinc homeostasis has been investigated mainly in *S. cerevisiae*: Following the pattern of a high proportion of zinc-binding proteins in eukaryotes, about 8% of the yeast proteome is thought to bind zinc (Andreini *et al.* 2006b) and more than 400 yeast genes are involved in growth under zinc limitation (North *et al.* 2012). These include genes essential for zinc homeostasis, but also endoplasmic reticulum (ER) function, oxidative stress resistance, protein folding, vesicular trafficking and chromatin modification. Moreover, SODs, which are essential for the detoxification of reactive oxygen species (ROS) generated by host cells, are copper-, manganese- and zinc-dependent enzymes (Huang *et al.* 2009).

Consequently, zinc is vital for growth and metabolism in both the host and pathogens. Thus, like for iron, there is a constant competition for zinc during infections, and zinc sequestration is another aspect of the vertebrates' nutritional immunity (Corbin *et al.* 2008). The frequently near-neutral pH in the host lowers the solubility of zinc and therefore restricts its accessibility for microorganisms. In the oral cavity, antimicrobial peptides within saliva, the histatins, are able to bind zinc and copper, which adds to their inhibitory effect on the growth of *C. albicans* (Gusman *et al.* 2001). Intracellularly, stimulated T cells, macrophages and dendritic cells decrease their lysosomal zinc content via the expression of the zinc transporter ZIP8, inducing zinc limitation for pathogens in the phagolysosome (Begum *et al.* 2002; Aydemir *et al.* 2009). Similarly, stimulated dendritic cells reduce their cytoplasmic zinc concentration by upregulating zinc exporters and downregulating zinc importers (Kitamura *et al.* 2006). Cytokine-activated macrophages restrict the intracellular growth of *H. capsulatum* by diminishing intracellular zinc availability (Winters *et al.* 2010) via binding to MTs and by sequestering labile zinc into the Golgi apparatus (Vignesh *et al.* 2013).

The host protein calprotectin inhibits bacterial and fungal growth by chelating transition metals, including zinc (Lulloff, Hahn and Sohnle 2004; Corbin *et al.* 2008). In fact, calprotectin is the most abundant cytosolic protein of neutrophils and is released mainly during the formation of neutrophil extracellular traps (NETs) as their key antifungal effector (Urban *et al.* 2009; Bianchi *et al.* 2011). *In vitro* the stimulation of neutrophils with phorbol myristate acetate triggers NET formation, which leads to the reduction of the supernatant zinc content, while no changes were detected for Fe, Cu and Mn concentrations (Niemiec *et al.* 2015). NET-dependent inhibition of fungal growth is consequently reversible *in vitro* by zinc supplementation (Urban *et al.* 2009; McCormick *et al.* 2010; Bianchi *et al.* 2011).

Fungi have developed sophisticated countermeasures to this host-imposed zinc limitation, including the expression of high-affinity membrane zinc importers and specialized secreted zinc uptake proteins, known as zincophores, in order to obtain zinc from the host environment (Citiulo *et al.* 2012). However, excessive zinc levels can also be toxic for cells—mainly due to competition with other metals for metal-binding sites in enzymes (McDevitt *et al.* 2011; Gu and Imlay 2013), as zinc does not participate in Fenton chemistry. Vertebrates use this to their advantage and are able to accumulate zinc to toxic levels in certain niches. As an example from bacteria, a drastic increase of the intraphagosomal zinc level leads to an impaired growth of *Mycobacterium tuberculosis*, although the bacterium can partially cope with this metal excess by the expression of metal efflux ATPases (Botella *et al.* 2011).

Zinc homeostasis and uptake

Our knowledge of zinc transporters, their transcriptional regulation and zinc trafficking mechanisms within the cell (Fig. 2) is (again) based, for a good part, on studies in *S. cerevisiae*—all these were first described in baker's yeast. There are two known classes of eukaryotic zinc transporters: ZRT-IRT-like proteins (ZIP) (Grotz *et al.* 1998), which include *S. cerevisiae* Zrt1, Zrt2 and Zrt3 (MacDiarmid, Gaither and Eide 2000), and the cation diffusion facilitators (Paulsen and Saier 1997), represented by Zrc1, Cot1, Msc2 (Li and Kaplan 2001) and Zrg17 (Ellis, Macdiarmid and Eide 2005).

The uptake of zinc from the extracellular milieu takes place mainly via two ZIP transporters in *S. cerevisiae*, the high-affinity Zrt1 (Zhao and Eide 1996a) and the low-affinity Zrt2 membrane transporters (Zhao and Eide 1996b). Under severe zinc limitation, *ZRT1* expression increases 30-fold (Zhao and Eide 1996a) compared to optimal zinc conditions, while *ZRT2* is usually expressed only under mild zinc limitation. In addition, under conditions of low zinc, *S. cerevisiae* also expresses the low-affinity metal transporter Fet4 that imports zinc, iron and copper into the cell (Li and Kaplan 1998). An additional system that exists is the phosphate/H^+ symporter family member Pho84, a known phosphate transporter, which is also able to import zinc complexed with phosphate (Jensen, Ajua-Alemanji and Culotta 2003).

Aspergillus fumigatus is able to robustly grow under a broader range of pH values than *S. cerevisiae*, especially in alkaline environments (Wheeler, Hurdman and Pitt 1991; Amich *et al.* 2010) where metal solubility is low (Martinez and Motto 2000). Of its eight putative ZIP transporters, ZrfA and ZrfB have functions in zinc uptake that resemble *S. cerevisiae* Zrt1, although ZrfB appears to be the main transporter (Vicentefranqueira *et al.* 2005). Interestingly, and in contrast to baker's yeast, this system is active only under acidic pH (Vicentefranqueira *et al.* 2005). In neutral to alkaline low zinc environments, resembling host tissue, *A. fumigatus* instead employs the ZrfC zinc transporter, which does not have a *S. cerevisiae* ortholog (Amich *et al.* 2010). Its ability to acquire zinc in alkaline environments seems to depend on its long N-terminus (not present in ZrfA and ZrfB), which contains four putative zinc-binding motifs (Amich *et al.* 2010). Consequently, this N-terminal sequence was found to be important for zinc uptake during lung infections, and it enables growth

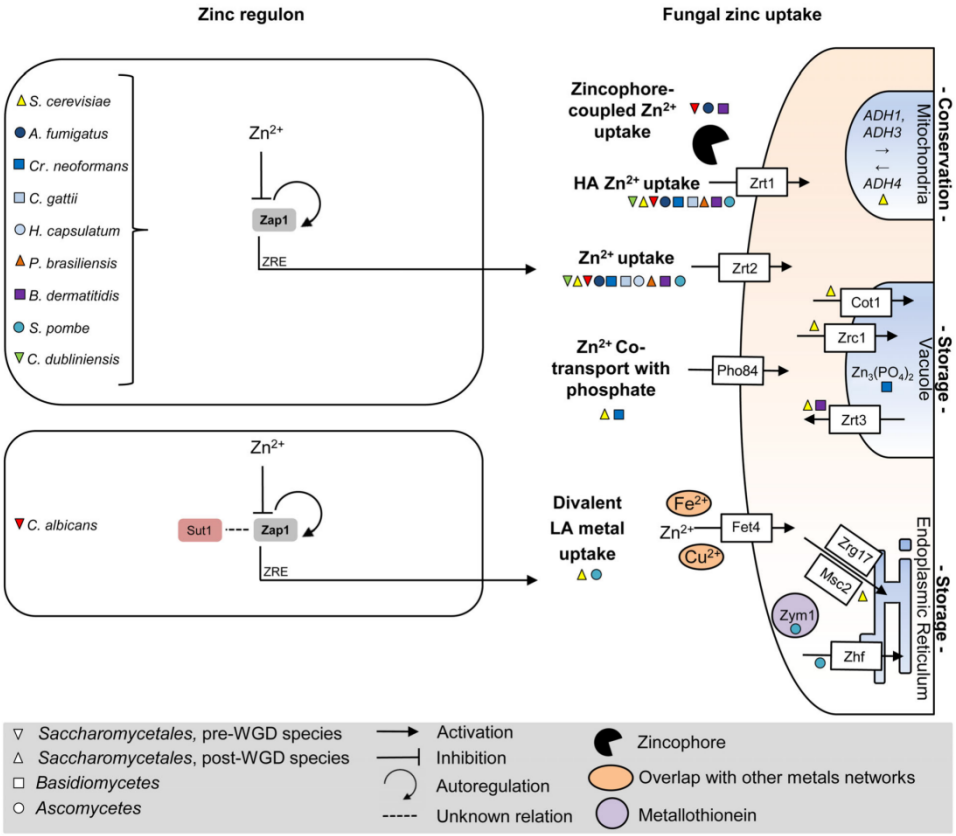

Figure 2. Fungal zinc homeostasis. Regulation of zinc homeostasis (left panel side) is shown for different fungal species (species is color coded, shape defines phylogenetic ancestry according to Gabaldon *et al.* 2013). Major transcription factors upregulated during zinc starvation to initiate fungal zinc uptake (right panel side) are written in bold. Orthologs are color shaded and aligned vertically. ZRE, recognition of target genes via zinc responsive elements. HA, high affinity; LA, low affinity.

even in the presence of zinc-binding calprotectin (Amich *et al.* 2014).

The *Cr. neoformans* and *Cr. gattii* zinc uptake systems comprise the ZIP transporters Zip1 and Zip2, orthologs of *S. cerevisiae* Zrt1 and Zrt2, respectively (Do *et al.* 2016). In both fungi, the high-affinity membrane transporter Zip1 is the main (pH-independent) zinc importer, while Zip2 seems to contribute little, if anything, to zinc uptake *in vitro* (de Oliveira Schneider *et al.* 2015; Do *et al.* 2016). In *Cr. gattii*, both transporters must be deleted for a visible effect on virulence (de Oliveira Schneider *et al.* 2015), while in *Cr. neoformans*, deletion of Zip1 already results in attenuation in a mouse model of cryptococcosis (Do *et al.* 2016). However, residual virulence even in a *Cr. neoformans zip1*Δ*zip2*Δ double deletion mutant hints towards additional, still undetected zinc uptake mechanisms in this fungus and possibly, *Cr. gattii*. Interestingly, a connection between phosphate uptake and zinc homeostasis was shown for *Cr. neoformans* (Kretschmer *et al.* 2014), which could imply a role of its Pho84 homologs in zinc uptake similar to *S. cerevisiae*. Further zinc-regulated homologs of Zrt1 and/or Zrt2 have been described in *H. capsulatum* (Dade *et al.* 2016), *P. brasiliensis* (Parente *et al.* 2013) and *B. dermatitidis* (Muñoz *et al.* 2015), generally in connection to virulence—indicating the central role of zinc and this conserved acquisition system in fungal diseases.

Not surprisingly, *C. albicans* follows the same pattern of transport via Zrt1 and Zrt2 ZIPs (Kim *et al.* 2008), and again zinc uptake was found to be upregulated in the early stages of *C. albicans* infection in mice (Xu *et al.* 2015). However, the *C. albicans* zinc uptake system was shown to additionally include a 'zincophore' (Citiulo *et al.* 2012). In response to alkaline pH and to zinc limitation, *C. albicans* releases the metalloprotease-like Pra1 into the medium, where it is able to bind zinc ions with high affinity. Zinc-loaded Pra1 can then bind back to Zrt1, in a manner reminiscent of siderophores used by other fungi for iron (Citiulo *et al.* 2012). Interestingly, *PRA1* and *ZRT1* are co-expressed (Ihmels *et al.* 2005), as they share the same upstream intergenic region, and both were found to be upregulated on epithelial cells and in a liver infection model (Thewes *et al.* 2007; Zakikhany

et al. 2007). So far, the *C. albicans* Pra1-Zrt1 pairing is the only proven zincophore system in fungi, but a similar locus structure is conserved in *A. fumigatus*: *ASPF2-ZRFC* is orthologous to *PRA1-ZRT1* (Amich *et al.* 2010), and like Pra1, AspF2 is secreted in high amounts during infections (Segurado *et al.* 1999). Not surprisingly, a possible zincophore function has recently been suggested (Amich *et al.* 2014). In *B. dermatitidis* mice infections, *BDFG_05357* is one of the most highly expressed genes. Like Pra1, it encodes an HRXXH domain-containing secreted protein, and has also been predicted to function as a zincophore (Muñoz *et al.* 2015). It seems that research into zincophores and their role in fungal pathogenesis is still gathering momentum.

High zinc levels can pose the opposite problem, and surplus zinc must be dealt with swiftly by the microorganism. In fungi, the vacuole serves as an organelle for zinc sequestration, storage and detoxification. Vacuolar zinc homeostasis has been investigated in some detail in *S. cerevisiae*, where it depends—among others—on the Zrc1 and Cot1 zinc importers of the vacuolar membrane (MacDiarmid, Gaither and Eide 2000). Surprisingly, *ZRC1* transcription is also induced under low zinc concentration, likely in anticipation of a possible sudden zinc excess: as all zinc importers are fully active, they will immediately relay any environmental increase in zinc abundance (MacDiarmid, Milanick and Eide 2003). Inside the vacuole, zinc is likely bound to polyphosphates, as shown for *Cr. neoformans* (Kretschmer *et al.* 2014). In contrast, *Sc. pombe* does not rely on the vacuole as a zinc sink; instead, the zinc homeostasis factor, Zhf, transports excess zinc into the ER (Borrelly *et al.* 2002; Clemens *et al.* 2002)—a function derived maybe from its *S. cerevisiae* counterpart, Msc2, which in a heterodimer with Zrg17 imports zinc into the ER for proper protein processing (Li and Kaplan 2001; Ellis *et al.* 2004). *Schizosaccharomyces pombe* strikingly also uses the metallothionein Zym1 to sequester zinc, similar to higher eukaryotes, but in contrast to other fungi, where MTs mainly sequester copper (Borrelly *et al.* 2002).

The vacuole not only serves as an emergency disposal site, but can also replenish cellular zinc in times of need. Zinc mobilization under starvation occurs via the Zrt3 vacuole zinc exporter in *S. cerevisiae* (MacDiarmid, Gaither and Eide 2000). Its orthologs have been found upregulated during co-incubation of *B. dermatitidis* with macrophages (Muñoz *et al.* 2015) and during zinc starvation in *C. dubliniensis* (Böttcher *et al.* 2015). Another approach to deal with low zinc is to conserve the metal by decreasing its use. *S. cerevisiae* reduces the expression of major zinc-dependent enzymes and induces expression of alternative proteins of identical function, which either require less zinc or different metals. For example, the alcohol dehydrogenases Adh1 and Adh3 (which bind two zinc ions each) are replaced under zinc limitation by Adh4, which only requires one zinc ion, allowing cells to continue fermentation even under zinc deficiency (Bird *et al.* 2006). Important infection-associated extracellular SODs of *C. albicans* (Sod4–6) and *H. capsulatum* (Sod3) uniquely use a single copper instead of the otherwise nearly universal Cu and Zn cofactors of SODs, likely reflecting the copper-rich, zinc-poor host environment (Gleason *et al.* 2014a)—a factor we will come back to in the section on copper.

Zinc sensing and transcriptional regulation

In contrast to iron and copper, zinc is a redox-inactive metal and does not damage cells via ROS. However, it avidly binds to many metallation sites of proteins, replacing the native metal and interfering with their function. Hence, like for the other metals, zinc homeostasis must be precisely regulated. In yeast, the zinc responsive activator protein 1 (Zap1) is the major transcription factor regulating zinc homeostasis genes (Zhao and Eide 1997). It binds to conserved zinc responsive elements in the promoters of more than 80 genes, including *ZRT1*, *ZRT2*, *ZRT3*, *FET4* and *ZRC1* (Wu *et al.* 2008). Moreover, Zap1 positively autoregulates its own expression to ensure a robust response to zinc limitation (Zhao and Eide 1997; Wu *et al.* 2008). The structure of Zap1 was analyzed in detail in *S. cerevisiae*: it contains two activation domains, AD1 and AD2, which are evolutionary conserved within the fungal species (Frey and Eide 2011); AD1 is responsible for the induction of most Zap1 target genes, while AD2 regulates genes when zinc deficiency appears in concert with other stresses (Frey and Eide 2011). The intracellular zinc level is sensed via direct interaction of metal and protein: under a sufficient cytosolic zinc concentration, zinc ions directly bind AD1 and AD2 to inhibit the expression of Zap1 targets (Frey and Eide 2011). Overall, this system is highly conserved within fungi and can be found with few variations throughout the non-pathogenic and pathogenic species, including *Cr. gattii* (Zap1, de Oliveira Schneider *et al.* 2012) and *A. fumigatus* (ZafA, Moreno *et al.* 2007), and in both it was found important for full virulence.

For a fast downregulation of the importers during zinc repletion, post-translational effects come into play. Zrt1 is a stable membrane protein under low environmental zinc levels; however, the presence of zinc leads to its rapid ubiquitination and internalization for vacuolar degradation (Gitan *et al.* 1998). Moreover, under low zinc, Zap1 activates the expression of *PIS1*, encoding a phosphatidylinositol synthase, and *DTT1*, encoding a diacylglycerol pyrophosphate phosphatase, which results in increased levels of phosphatidylinositol and decreased levels of phosphatidylethanolamine in the membrane (Carman and Han 2007). This change in the membrane phospholipid composition is thought to influence the function and the localization of membrane zinc transporters.

The *C. albicans* Zap1 ortholog, also called Csr1, controls zinc homeostasis including Pra1 expression (Nobile *et al.* 2009), but is, of note, also involved in filamentation and biofilm matrix elaboration (Kim *et al.* 2008; Nobile *et al.* 2009)—two important contributors to *C. albicans* virulence. However, the virulence defect of a *csr1*Δ mutant likely depends not only on these morphological effects, but also directly on defective zinc homeostasis in the host. In support of this, a *csr1*Δ mutant of the closely related species *C. dubliniensis* shows no such filamentation defects, but still exhibits reduced virulence (Böttcher *et al.* 2015). Interestingly, in *C. albicans*, an additional transcription factor, Sut1, was recently implicated in controlling Csr1 expression *in vivo*, but surprisingly not *in vitro* (Xu *et al.* 2015). No functional relationship between the two *S. cerevisiae* counterparts is known (Xu *et al.* 2015), which suggests that this seemingly host-specific interaction is an adaptation to the pathogenic lifestyle of *C. albicans*. It will be interesting to see whether any other pathogen exhibits a similar departure from *S. cerevisiae*'s zinc regulation template.

A final twist is the pH-dependency of zinc uptake. As mentioned before, *A. fumigatus* switches from zinc uptake via ZrfA and ZrfB to ZrfC (and possibly AspF2) depending on the environment's alkalinity. While the Zap1 ortholog ZafA activates all transporters under zinc limitation independent of pH, the pH-dependent transcription factor PacC represses ZrfA and ZrfB under alkaline pH (Amich, Leal and Calera 2010) and ZrfC/AspF2 under acidic conditions (Amich *et al.* 2010). In *C. albicans*, expression of the Zrt1/Pra1 zincophore is similarly alkaline specific via the PacC ortholog Rim101 (Bensen *et al.* 2004; Citiulo *et al.* 2012; Xu *et al.* 2015), mirroring the Rim101-dependent expression of

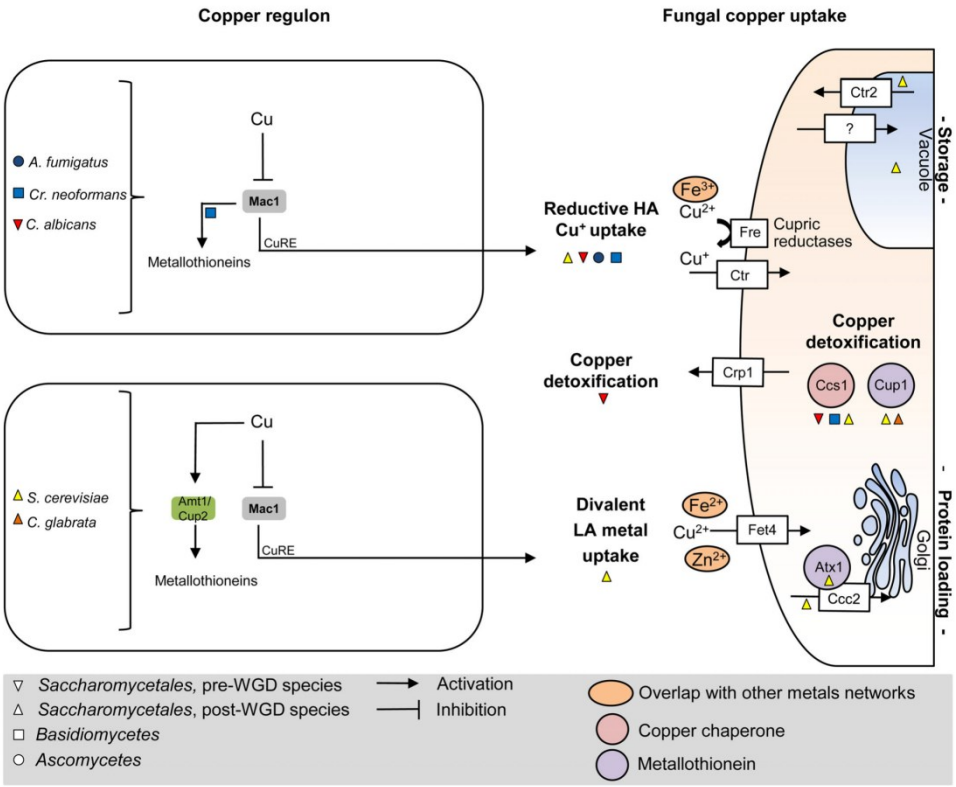

Figure 3. Fungal copper homeostasis. Regulation of copper homeostasis (left panel side) is shown for different fungal species (species is color coded, shape defines phylogenetic ancestry according to Gabaldon *et al.* 2013). Major transcription factors upregulated during copper starvation to initiate fungal copper uptake (right panel side) are written in bold. Orthologs are color shaded and aligned vertically. CuRE, recognition of target genes via copper responsive elements. HA, high affinity; LA, low affinity.

iron uptake-related genes. It seems likely that these expression patterns evolved as highly effective systems to deal with the low solubility of metals under alkaline conditions.

COPPER

Copper is in many ways a different beast than iron or zinc (Fig. 3). Like those metals, it is required as an essential trace element in many biochemical reactions, but it rapidly becomes highly toxic at increased levels (reviewed in Festa and Thiele 2011). Copper started to be bioavailable at a large scale only after the great oxidation event ≈2.4 billion years ago, when earth's atmosphere became oxidizing. Eukaryotes, which evolved after these events, consequently harbor many more Cu-containing proteins than the more ancient bacteria (Dupont *et al.* 2010). For the same reason, many Cu-containing enzymes have oxygen-related functions. For instance, the mitochondrial cytochrome c oxidase requires Cu for its function in the respiratory electron transport chain. Cytoplasmic or cell-wall associated Cu/Zn-SODs (like their mostly mitochondrial manganese-dependent counterparts) can protect fungal cells from externally and internally generated oxidative stress. Again, the *C. albicans* SODs are unusual: *C. albicans* is the only known organism to contain both Cu/Zn- and Mn-SOD enzymes in the cytosol (Lamarre *et al.* 2001)—in addition to the Cu-only variety of extracellular SODs mentioned above. The Mn-dependent Sod3 is expressed to replace the Cu-dependent counterparts under copper starvation, for example during infections of the murine kidney (Li *et al.* 2015). This flexibility probably tells as much about the necessity of SODs for pathogens as about the diverse metal environments *C. albicans* is facing during infections. In addition, copper has an important helper role as a cofactor in multicopper ferroxidases to allow the uptake of iron via the reductive pathway (see above). Finally, it also has an important function as a cofactor of laccases and tyrosinases (Shaw and Kapica 1972; Williamson 1994), which are required for the biosynthesis of melanin—an important virulence factor of pigmented fungi.

However, due to its toxicity, copper has also been used as an antimicrobial agent for much of human civilization. As a fungicide against plant pathogens, it is part of the Bordeaux mixture used in vineyards, and copper surfaces show promise

as a weapon against pathogens in hospitals (Casey *et al.* 2010). Part of its toxic effects derives from the ability of Cu^{+} (under anaerobic, reducing conditions) to disrupt Fe-S clusters (Macomber and Imlay 2009) and from its high capacity to displace other metals from their coordination sites, as, according to the Irving-Williams series, Cu^{2+} forms the most stable complexes of the divalent transition metals (Irving and Williams 1948). Furthermore, like iron, it can also readily form ROS by the Fenton reaction by Cu^{+}/Cu^{2+} redox cycling under aerobic conditions, although the precise role of this for microbes is somewhat disputed (Macomber, Rensing and Imlay 2007), and in fact copper seems even more toxic under anaerobic than under aerobic conditions both for bacteria (Evans *et al.* 1986) and fungi like *S. cerevisiae* and *C. albicans* (Strain and Culotta 1996; Weissman, Shemer and Kornitzer 2000).

Given this comparatively high toxicity, the host and fungal strategies during infections differ significantly from the Fe-based nutritional immunity: instead of limiting access, the host seems to actively pump copper into microbe-containing phagosomes via the P-type ATPase ATP7A (Wagner *et al.* 2005; White *et al.* 2009). In fact, *Cr. neoformans* copper detoxification is activated during murine pulmonary infections, and the relevant MTs are required for virulence in this model (Ding *et al.* 2013). According to some reports, copper limitation may also play a role as an immune defense mechanism. A *Cr. neoformans* copper transporter was seen to be upregulated after phagocytosis by macrophage-like cells and during human cryptococcosis (Waterman *et al.* 2007, 2012), and the *C. albicans* copper transporter similarly shows upregulation upon phagocytosis (Lorenz, Bender and Fink 2004). Whether these observations represent a bona fide copper limitation or a loss of bioavailability due to the oxidative phagosomal environment (Waterman *et al.* 2007) remains to be seen. However, overlapping regulation of Cu uptake and resistance pathways (Ding *et al.* 2011), as well as possible confounding effects of the deletion and detection systems, seem to call for further investigation into the matter (Ding *et al.* 2013). Thus, the jury is still out whether both Cu ion overload and withholding are complementary strategies employed by the host, possibly depending on the microenvironment the fungus is facing.

Copper homeostasis and uptake

Similar to iron, copper is usually reduced from Cu^{2+} to Cu^{+} (in part by the same cell-surface metalloreductases as for Fe) for efficient uptake and then imported via high-affinity Cu^{+} importers—Ctr1 in *C. albicans* (Marvin, Williams and Cashmore 2003), the functionally redundant Ctr1 and Ctr4 in *Cr. neoformans* (Ding *et al.* 2011), and at least two importers (CtrA2 and CtrC) in *A. fumigatus* (Park *et al.* 2014). In contrast to iron, no oxidase is involved in this process. In *S. cerevisiae* at least, the iron transporter Fet4 also imports copper with low affinity (Hassett *et al.* 2000). Another source of copper in addition to the surrounding medium is the vacuolar storage. In *S. cerevisiae*, the transmembrane copper transporter Ctr2, a homolog of Ctr1, allows copper mobilization from this organelle (Rees, Lee and Thiele 2004) with the help of a metalloreductase in the vacuolar membrane (Rees and Thiele 2007), mimicking the cytoplasmic membrane setup. Pathogenic fungi like *C. albicans* possess orthologs of these proteins, but their role in virulence has not been investigated so far.

Once intracellular, the potentially toxic Cu^{+} is immediately bound by different specific chaperones, which allow its quick and targeted transport to Cu-requiring enzymes. For example, Ccs1 proteins deliver copper to the Cu/Zn-SODs of *C. albicans* (Gleason *et al.* 2014b), *Cr. neoformans*, *S. cerevisiae* (Liu *et al.* 1997) and in fact nearly all eukaryotes (Leitch *et al.* 2009). Similarly, Atx1 homologs escort copper to Ccc2 Cu-transporting ATPases of the Golgi membrane (Lin *et al.* 1997; Huffman and O'Halloran 2000). These then pump the metal into late secretory vesicles to serve as a cofactor, for example, in the aforementioned Fe multicopper oxidases or laccases. This also intimately links copper to iron homeostasis, as multicopper oxidases are required for efficient iron uptake in fungi like yeast or *C. albicans* (Askwith *et al.* 1994; Eck *et al.* 1999; Cheng *et al.* 2013).

A similar binding mechanism prevents toxicity under high copper conditions. MTs, small proteins rich in cysteine residues, can sequester Cu (and, especially in non-fungal organisms, other metals) to render it biologically inactive. They are also present in plants and animals, but in very few bacteria—one example being specifically the pathogenic mycobacteria (Gold *et al.* 2008). Characteristically, the genes coding for MTs vary strongly in numbers between species: in pathogenic fungi, some *C. glabrata* strains harbor more than 30 copies of the MT-IIa gene, in addition to one copy each of MT-IIb and MT-I (Mehra, Garey and Winge 1990; Mehra *et al.* 1992). Similarly, *S. cerevisiae* can increase its copy number of the *CUP1* metallothionein gene and thereby obtain higher Cu resistance (Fogel and Welch 1982). No such mechanism has been described for *C. albicans* with its three known MTs or *C. neoformans* with its two (Ding *et al.* 2011) so far. Similarly, it seems that in *S. cerevisiae* copper is also detoxified, like other metals, via the vacuolar storage (Szczypka *et al.* 1997; Jo *et al.* 2008), but little is known about this process in other fungi.

In *S. cerevisiae* (and likely other fungi), high intracellular Cu levels furthermore rapidly block the Ctr1 Cu importer by direct binding and subsequent conformational changes to restrict copper influx (Wu *et al.* 2009). However, *C. albicans* achieves its high intrinsic Cu resistance (when compared to *S. cerevisiae*) also by active outward transport over the plasma membrane by Crp1, a P-type ATPase (Riggle and Kumamoto 2000; Weissman *et al.* 2000), in a process functionally resembling the copper transport by Ccc2 ATPase into the Golgi (Weissman, Shemer and Kornitzer 2002)—or even into the phagosome by the host's ATP7A, in an interesting example of a molecular-level arms race using the same mechanism on both sides. This export mechanism, although common in bacteria (reviewed in Samanovic *et al.* 2012) and present in other eukaryotes, has so far been found only in *C. albicans* and—very recently—in *A. nidulans* (Antsotegi-Uskola, Markina-Inarrairaegui and Ugalde 2017).

Copper sensing and transcriptional regulation

Low copper levels lead to an activation of the transcription factor Mac1 in *S. cerevisiae* (Jungmann *et al.* 1993), and the same is true for its orthologs in *C. albicans* (Mac1; Marvin, Mason and Cashmore 2004), *A. fumigatus* (Afmac1; Kusuya *et al.* 2017) and most likely also *C. glabrata*. The Mac1 activator comprises a copper fist DNA-binding domain to recognize copper response elements, and a Cu-binding domain to gauge the intracellular copper concentration and inhibit DNA binding under copper replete conditions (Graden and Winge 1997). Under copper starvation, Mac1 binding leads to the expression of the dedicated copper transporter and metalloreductase genes via their upstream regulatory elements (Yamaguchi-Iwai *et al.* 1997). Under copper excess, Mac1 is quickly degraded to avoid copper toxicity (Zhu *et al.* 1998), and in contrast to copper-depleted conditions, *MAC1* mRNA exists in a readily degradable isoform when copper

is present (Peccarelli *et al.* 2016). This Cu-dependent regulation directly influences virulence: deletion of the Mac1 ortholog Cuf1 reduces dissemination of *C. neoformans* to the mouse brain, and abolishes transcription of the copper-dependent laccase (Jiang *et al.* 2009). In *C. albicans*, Mac1 is—among other functions—responsible for shifting from the Cu-dependent Sod1 to the Cu-independent Sod3, by repressing the former and activating the latter (Li *et al.* 2015).

The *Cr. neoformans* Cuf1 (Lin *et al.* 2006; Waterman *et al.* 2007) is not only responsible for upregulation of copper uptake under starvation, but also positively regulates MTs under Cu excess (Ding *et al.* 2011). In fact, Cuf1 seems to be a hybrid factor, as in *C. glabrata* and *S. cerevisiae* these roles are separated, and in *C. glabrata* another transcription factor, called Amt1 [homologous to Cup2 or Ace1 in *S. cerevisiae* (Buchman *et al.* 1989; Szczypka and Thiele 1989)], is activated under high copper levels by the binding of four Cu^{+} ions to its N-terminal domain (Thorvaldsen *et al.* 1994). Active Amt1 then induces the transcription of all three MT genes and itself, leading to a positive autoregulatory loop and thus a robust copper resistance response (Zhou *et al.* 1992; Zhou and Thiele 1993; Koch *et al.* 2001). The role of its homolog in *C. albicans* is not well investigated so far (although it likely has similar functions), but the cAMP pathway has been implicated in copper resistance in this fungus. A deletion of *C. albicans GPA2* (encoding the G-protein α subunit upstream of protein kinase A) decreases Cu uptake, increases MT expression and hence renders the fungus more resistant to copper (Schwartz *et al.* 2013). Overall, the typical fungal response to high copper thus seems to be determined by a fast inactivation and degradation of the Mac1 activator homologs, and copper sequestration via upregulation of MTs by different mechanisms. However, our knowledge of these regulatory systems still lacks behind what we have learned about zinc and especially iron homeostasis in fungal pathogenesis.

NICKEL

Nickel is a comparatively rare metal, but an efficient fungicide that seems to exert its effects mainly by interfering with the carbohydrate metabolism and DNA repair, by production of ROS (albeit less than copper or iron), and by membrane damage (reviewed in Macomber and Hausinger 2011). Many of these effects are exerted by nickel replacing the original metal in metalloenzymes—and as nickel is rather stable in the Ni^{2+} state, this replacement abolishes the redox function of the metal cofactor (Macomber and Hausinger 2011). At high external concentrations, nickel can non-specifically enter the microbial cell via the magnesium transport system. Still, dedicated uptake systems for this mostly toxic transition metal also exist, especially in bacteria (Zhang *et al.* 2009), and a functional Ni permease with high similarity to its bacterial co-family members has, for example, been found in *Sc. pombe* (Eitinger *et al.* 2000). So why would microbes, and especially fungi, actively import nickel? In *Sc. pombe*, this seems to be related to its urease activity (Eitinger *et al.* 2000), which requires Ni to allow the use of urea as a nitrogen source and the concomitant alkalization of the environment. For pathogens, ureases (and with them, most likely dedicated nickel permeases) often play important roles as virulence factors, for example, in *Coccidioides immitis* and in *Cr. neoformans* (Singh *et al.* 2013). With no known Ni metalloenzymes in vertebrates, nickel homeostasis has thus been suggested as a promising avenue for fighting infections (Morrow and Fraser 2013). However, the *Saccharomycetes*—like *S. cerevisiae*, *C. albicans* and *C. glabrata*—do not employ a Ni-requiring urease (Navarathna *et al.* 2010), and consequently seem to lack Ni permeases—instead, these fungi use non-nickel, biotin-requiring urea amidolyases to metabolize urea (Navarathna *et al.* 2010). In *A. fumigatus*, a nickel permease homolog can be found in the genome, but little is known so far about its potential role in virulence.

Excess nickel, as is so often the case with toxic metals, is sequestered into the vacuole by *S. cerevisiae* (Nishimura, Igarashi and Kakinuma 1998)—in this case with the help of the avid nickel binder, histidine (Pearce and Sherman 1999). It seems likely that pathogenic fungi have similar mechanisms at their disposal, paralleling the existence of nickel resistance mechanisms in many bacteria. Overall, however, little is currently known about the role of nickel in fungal pathogenesis, and we may yet be surprised by unexpected findings in the future.

MANGANESE

Manganese is required in the function of polymerases, sugar transferases of the Golgi and of course for the Mn-SODs especially of the mitochondria (reviewed for baker's yeast in Reddi, Jensen and Culotta 2009). Its intracellular concentration has been shown to vary significantly, over nearly two orders of magnitude (Reddi, Jensen and Culotta 2009). One reason may be that—in contrast to most of the other metals described here—manganese acts as an anti-oxidant at high concentrations, rather than a ROS producer. In fact, at high intracellular concentrations Mn-containing complexes can take the role of SODs in certain bacteria and in yeast SOD deletion mutants (Reddi *et al.* 2009). Excessive levels are nonetheless toxic to yeasts leading to the induction of apoptosis (Liang and Zhou 2007).

External manganese is taken up in baker's yeast via the Nramp transporters, Smf1 and Smf2 (Supek *et al.* 1996; Cohen, Nelson and Nelson 2000; Portnoy, Liu and Culotta 2000), and a possible ortholog in *C. neoformans* has been described to transport Mn and other metals (Agranoff *et al.* 2005). It has been suggested that Smf1 is responsible for keeping up the intracellular Mn levels required for its anti-oxidant action, while Smf2 imports manganese for the Mn-requiring enzymes (Luk and Culotta 2001; Reddi *et al.* 2009). These transporters are continuously expressed and regulated mainly post-translationally, and at sufficiently high (physiological) Mn levels they are continually targeted for vacuolar degradation (Reddi *et al.* 2009). Furthermore and in a manner similar to zinc, high extracellular manganese can be imported by yeast in complex with phosphate via the Pho84 transmembrane transporter (Jensen, Ajua-Alemanji and Culotta 2003). Once inside the cell, it can then be transported by the Golgi P-type Ca^{2+}/Mn^{2+} ATPase, Pmr1, to serve as a cofactor in the secretory pathway (Dürr *et al.* 1998). In fact, a Pmr1 homolog is required for full *C. albicans* virulence due to this cofactor role in glycosylation (Bates *et al.* 2005). Finally, in *S. cerevisiae* at least, excess manganese is excreted via the secretory pathway, but also sequestered to the vacuole (like iron via Ccc1; Li *et al.* 2001), and in *C. albicans* its complexation with polyphosphate has been shown (Ikeh *et al.* 2016). If and how manganese can leave the vacuole again is still an open question, as no dedicated exporter has been described so far.

Due to these biological functions, the host employs Mn starvation to fight bacteria and possibly fungi. Macrophage phagosomes are severely limited for manganese (Jabado *et al.* 2000), and the host-defense protein, calprotectin, chelates manganese

in addition to zinc (Corbin *et al.* 2008) and—as shown recently—iron (Nakashige *et al.* 2015). *In vitro* at least, Mn chelation by calprotectin reduces growth of *A. fumigatus* (Amich *et al.* 2014; Clark *et al.* 2016), and Mn withdrawal may thus play a role in fungal infections—although in contrast to bacteria, the effects of manganese limitation on fungal virulence are probably eclipsed by the removal of zinc and iron. As with nickel, research into the role of manganese in fungi may yet reveal some unexpected connections to pathogenesis, as our knowledge so far is comparatively incomplete.

CONCLUSIONS

Metals clearly play a central role during fungal pathogenesis. This is shown by the sheer number and diversification of the regulatory, uptake and detoxification systems in fungal pathogens, and of course by the host's many efforts to efficiently withhold metals. We seem to have a good concept of how iron and—with a few gaps—zinc are acquired by fungi during infections, but for many of the metals that are experimentally more difficult to address, our knowledge is still quite limited. The protection mechanisms against many metals with toxic effects are not well established, nor are the uptake systems for those which are required only in minute amounts—from cobalt to silver or cadmium. It seems likely that fungal research can learn a lot from the bacterial field, as even though the molecular mechanisms may differ, the basic problems the microbes are facing are essentially the same, and analogous solutions may have been found by both groups of pathogens.

Metal homeostasis also presents a largely untapped resource for potential treatment options. The natural response of the host already indicates the effectiveness of targeting the microbial requirement for metals. Strategies that may be worthwhile to follow in the future include a knowledge-guided combination of deprivation and excess: withholding one metal to induce a partially unspecific uptake response, which is exploited to introduce toxic levels of another. It seems that the immune system may already follow this strategy inside the phagosome, as described above e.g. for copper. Metal-based drugs were found highly effective against parasites like *Leishmania* spp. or *Plasmodium* spp. (reviewed in Navarro *et al.* 2010), and it seems at least possible that a similar approach may prove useful for fungi as well. We hope that with this review, we have enabled the reader to see the connections and similarities between metals and among fungi, maybe forming the kernel of a new hypothesis. The potential and the need for many more findings still exist in this growing field.

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5. Discussion

The challenges in treating *C. albicans* infections

The healthy human gut is colonized by multiple bacteria, archaea, fungi, protozoa, and viruses (including bacteriophages) (Sekirov, Russell et al. 2010). In recent years the relationship between the gut microbiota and human health has been increasingly recognized: The role of bacteria in the human gut was shown to be essential for various processes benefitting the host, including intestinal epithelial cell formation, extracting energy from non-digested food, host immune regulation, nutrient absorption, and vitamin synthesis (Bull and Plummer 2014, Thursby and Juge 2017). More recently, studies on the gut virome revealed beneficial effects of viruses on commensal bacteria within the gut (Kernbauer, Ding et al. 2014). However, the function of fungi, archaea, and protozoa in the human gut remains poorly investigated (Mai and Draganov 2009, Lozupone, Stombaugh et al. 2012, Huffnagle and Noverr 2013, Underhill and Iliev 2014, Iliev and Leonardi 2017).

C. albicans, a commensal yeast in healthy individuals, readily switches to a pathogenic stage and frequently causes superficial infections in immunocompromised patients. In severe cases, the fungus disseminates and invades the host's tissues. The treatment of disseminated *C. albicans* infections remains challenging, which is seen by a high morbidity and mortality rate (Brown, Denning et al. 2012). Currently a limited arsenal of antifungal drugs exists and fortunately, so far, the resistance of *C. albicans* to available antifungals is relatively low in comparison to other *Candida* species (Sanguinetti, Posteraro et al. 2015, Whaley, Berkow et al. 2016). Since both fungal and human cells are eukaryotes, there is a high degree of similarities in their structures. Therefore, some antifungals are also relatively toxic to humans and their side effects include organ damage and allergic reactions (Dixon and Walsh 1996, Kyriakidis, Tragiannidis et al. 2017). Additionally, *C. albicans* can form biofilms (on catheters, pacemakers, dentures, and prosthetic joints) that are resistant to both elevated concentrations of antifungals and the host's immunity (Nobile and Johnson 2015, Scorzoni, de Paula et al. 2017). Clearly, there is an urgent need for novel therapeutic approaches in *C. albicans* treatment. Since *C. albicans* is a common part of the gastrointestinal microbiota in healthy humans, it is likely to play a beneficial role in humans in its commensal state. Therefore the development of new drugs and vaccines against pathogen-specific virulence factors is an exciting strategy.

The host's nutritional immunity is able to both limit the availability of metals and increase their concentration in order to inhibit microbial growth. In turn, pathogens

employ various strategies to counteract both metals limitation and overload (Weinberg 1975, Botella, Peyron et al. 2011, Hood and Skaar 2012, VanderWal, Makthal et al. 2017). Both pathogens' requirement for transition metals and the toxicity of metals towards microorganisms are attractive areas for novel therapeutic approaches. This makes the pathogens' metal uptake and resistant systems a potential drug and/or vaccine target (Palmer and Skaar 2016).

Research into the effects of nutritional immunity in host-bacteria interactions has already led to the development of novel types of drugs. For example, gallium (Ga) and iron have an identical ionic radius, similar coordination chemistry (Shannon 1976, Bernstein 1998), and are barely discernible by biological systems (Kaneko, Thoendel et al. 2007, Nikolova, Angelova et al. 2016). In the absence of iron, gallium is able to substitute iron in its metalloproteins, including siderophores. However gallium is a redox inactive metal and such a replacement leads to the disturbance of vital redox-dependent reactions (Harrington, Martens et al. 2006). Gallium quenching of siderophores, produced by *Pseudomonas aeruginosa*, reduces the virulence of *P. aeruginosa in vivo* (Ross-Gillespie, Weigert et al. 2014). Importantly, during an experimental evolution test, *P. aeruginosa* rapidly developed resistance against the antibiotics typically used in clinical settings, but not against gallium loaded siderophores. Probably because evolving resistance against its own siderophores is not beneficial for *P. aeruginosa* (Ross-Gillespie, Weigert et al. 2014) Additionally, a Trojan horse strategy was successfully tested as an antibacterial treatment by coupling siderophores to antibiotics, such complexes are directly delivered to bacteria. In an interesting example of this technique, *Acinetobacter baumannii*, which is resistant to daptomycin (the drug does not penetrate through the bacterial cell wall), was treated with a conjugate of fimsbactin (a siderophore of *A. baumannii*) and daptomycin. This led to the active uptake of the siderophore-drug complex and a potent activity against *A. baumannii* both *in vivo* and *in vitro* (Ghosh, Miller et al. 2017).

To apply similar approaches to pathogenic fungi, more research on the regulation of metal homeostasis and effector mechanisms under fluctuating metal levels is needed. The availability of iron is recognized as a central factor during infections. After iron, zinc is the second most abundant transition metal in vertebrates and an essential cofactor for approximately 9% of proteins in eukaryotes (Andreini, Bertini et al. 2009). While *C. albicans* iron homeostasis and its response to external iron limitation is comparatively well investigated, the field of *C. albicans* zinc homeostasis has just recently started to receive significant attention. Therefore, the focus of the present thesis was to analyze two main aspects of *C. albicans* metal-related homeostasis strategies. Firstly, the regulation of iron homeostasis in response

to iron overload was characterized and compared to other fungal species. Secondly, the zinc homeostasis machinery and its contribution to *C. albicans* pathogenicity were investigated in detail.

Iron homeostasis

Fungi inhabit several ecological niches, and can be generally divided in environmental, commensal, opportunistic, and primary pathogenic species (Underhill and Pearlman 2015). The composition and availability of nutrients in the surrounding environment determines fungal development. The external iron concentration and its bioavailability drastically vary: In soil and within the host (during infections) iron has a low abundance (Weinberg 1975, Kehl-Fie and Skaar 2010, Xue, Xia et al. 2016), in the gastrointestinal tract the levels of iron are constantly changing (Chen, Pande et al. 2011, Kortman, Raffatellu et al. 2014), and in phagosomes the concentrations of iron might become elevated (VanderWal, Makthal et al. 2017). Thus, fungi, in order to obtain an optimal amount of iron and not to poison themselves with an overload, have evolved mechanisms to maintain iron homeostasis. Overall, the current knowledge of iron homeostasis in fungi indicates both specific and common strategies on dealing with iron fluctuations (Gerwien, Skrahina et al. 2018).

Among common trends in regulation of iron homeostasis in fungi is the presence of two TFs: a GATA TF for the repression of iron assimilation processes (Sfu1 in *C. albicans*, Cir1 in *C. neoformans*, SreA in *Aspergillus* spp. and *F. oxysporum*, Fep1 in *S. pombe,* and Urbs1 in *U. maydis*) and a CCAAT-binding complex TF for the repression of iron utilization processes (Hap43 in *C. albicans*, HapX in *C. neoformans, Aspergillus* spp., and *F. oxysporym*, Php4 in *S. pombe*, and Yap1 in *U. maydis*) (Voisard, Wang et al. 1993, Haas, Zadra et al. 1999, Oberegger, Schoeser et al. 2001, Tuncher, Sprote et al. 2005, Mercier, Pelletier et al. 2006, Hortschansky, Eisendle et al. 2007, Molina and Kahmann 2007, Schrettl, Kim et al. 2008, Jung, Saikia et al. 2010, Schrettl, Beckmann et al. 2010, Chen, Pande et al. 2011, Hsu, Yang et al. 2011, Lopez-Berges, Capilla et al. 2012, Kronstad, Hu et al. 2013).

Specific adaptations of fungi to iron fluctuations are highly likely to depend on the environment they face and include bi-functional and alternative regulators of iron homeostasis processes. For example, *A. fumigatus*, having both free living and pathogenic lifestyles, employs SreA, which represses iron acquisition processes under high iron levels (Schrettl, Kim et al. 2008), and directly interacts with HapX, which is an essential regulator under both iron starvation and excess conditions (Haas 2012, Gsaller, Hortschansky et al. 2014). Thus, gradually changing iron levels in the environment might benefit bi-functional receptors, as seen from *A. fumigatus*. In

contrast to *A. fumigatus* lifestyles, *C. albicans* is only present within the host. Although Hap43 in *C. albicans* and HapX in *A. fumigatus* share 41% amino acid sequence identity and both contain conserved domains (including CCAAT-binding, b(ZIP), coiled-coil domains, and cysteine-rich regions), the requirement of Hap43 for regulation of iron-dependent genes under high iron levels in *C. albicans* was not confirmed (Skrahina, Brock et al. 2017). The repression function of iron acquisition processes under elevated iron levels in *C. albicans* is limited to Sfu1 (Chen, Pande et al. 2011). Additionally, *C. albicans* integrates Sef1, an activator of iron assimilation processes, in the iron homeostasis network (Chen, Pande et al. 2011). *C. albicans* faces rapidly changing iron concentrations in its environment as a commensal yeast in the gut and a pathogenic fungus in various organs. Therefore, such a lifestyle favors a decisive, not overlapping, system to respond to deficient and sufficient iron levels. Thus the response is not intertwined by Hap43 and Sfu1, but possesses a reciprocal network (Chen, Pande et al. 2011, Skrahina, Brock et al. 2017).

The surrounding environment shaped the adaptation strategies of fungi to various iron levels. To this end, GATA type TFs were described for *H. capsulatum* (Hwang, Seth et al. 2012), *N. crassa* (Zhou, Haas et al. 1998), and *P. chrysogenum* (Haas, Angermayr et al. 1997). Since these species are both free living and pathogenic as *A. fumigatus*, the CCAAT type system is highly likely to also exist in these fungi. Interestingly, *C. glabrata* possesses an iron homeostasis regulation system that drastically differs from other pathogenic fungi and requires Sef1 and Aft1 TFs (Gerwien, Safyan et al. 2016). Such a system mostly mirrors *S. cerevisiae,* where Aft1 is a main positive iron regulator under iron limitation (Yamaguchi-Iwai, Dancis et al. 1995). However, the similarity of *C. glabrata* to the *C. albicans* system is seen in the requirement of Sef1 (Gerwien, Safyan et al. 2016). This similarity is in agreement with the observation that *C. glabrata* also appears mainly associated with the human host (Li, Redding et al. 2007, Presterl, Daxbock et al. 2007, Schmidt, Walker et al. 2008), and up until now it is not known if *C. glabrata* is present in the environment as a free living fungus. However, there are at least sporadic reports that identified the presence of *C. glabrata* in fruit juices (Deak 1993, Koc 2006). Such a possible association with fruits and a genetic similarity to *S. cerevisiae* (Muhlhausen and Kollmar 2014, Gabaldon and Carrete 2016) accompanied by the function of its iron regulation system suggests that *C. glabrata* may indeed be considered as a free living microorganism.

Interestingly, Hap43 homologs that regulate iron homeostasis are found exclusively in pathogenic fungi, except for *C. glabrata*. Thus, as a fungal-specific TF with a central function in virulence Hap43 is an attractive potential drug target. Since some of the currently available antifungals are toxic to humans, employing a unique

fungal TF as drug target would likely limit or avoid side effects on humans. The effectiveness of such a drug would probably be the highest against fungi, where Hap43 is required in both sufficient and deficient iron conditions (like *A. fumigatus*) and intermediate against species, where it is required only under deficient iron conditions (like *C. albicans*). In general, targeting of TFs can be highly effective, as these can disturb the regulation of several genes and often whole cellular functions, which are all regulated by the same TF. However the development of drugs that specifically target TFs remains challenging and so far such drugs were not administered in clinical settings (Bhagwat and Vakoc 2015).

Zinc homeostasis

Recently, the interest in the field of nutritional immunity has expanded beyond iron (Kehl-Fie and Skaar 2010). It has become evident that during host-pathogen interactions, there is a constant competition not only for iron, but also for manganese, cobalt, nickel, copper, and zinc (Palmer and Skaar 2016, Gerwien, Skrahina et al. 2018). However, while there are an increasing number of publications on the homeostasis of these metals, the accumulated knowledge is still lagging behind what we know about iron.

As previously mentioned, many of the fundamental biological discoveries, including metal homeostasis, were made in the model organism *S. cerevisiae*. Less is known for *C. albicans*, and therefore the present work focuses on the characterization of *C. albicans* zinc homeostasis on a global level by performing transcriptional and mutant screenings (manuscript in preparation Skrahina, Wilson et al.) and on a local level by analyzing single effector genes (Crawford, Lehtovirta-Morley et al. 2018). The initial analysis of the *C albicans* transcriptional response and the *C. albicans* transcription factor deletion library (Homann, Dea et al. 2009) screening revealed a high degree of overlap between *S. cerevisiae* and *C. albicans*. Both species under zinc deficiency conditions activate zinc uptake machinery, vacuolar zinc utilization, and oxidative stress resistance; and repress transcription, translation, metabolism, and biosynthesis processes (manuscript in preparation Skrahina, Wilson et al.) (Lyons, Gasch et al. 2000, Wu, Bird et al. 2008, North, Steffen et al. 2012). In addition, the *C. albicans* TF mutants *ace2*Δ/Δ, *swi4*Δ/Δ, *tup1*Δ/Δ, *sfl1*Δ/Δ, and *tye7*Δ/Δ were found to be growth defective under low zinc levels (manuscript in preparation Skrahina, Wilson et al.) and the orthologues of these TFs in *S. cerevisiae* also possess a zinc limitation phenotype (North, Steffen et al. 2012). So far, the role of these TFs in regulating the genes required for zinc homeostasis have not been investigated in *S. cerevisiae*.

The zinc uptake system in *S. cerevisiae* represents the high-affinity Zrt1 (expressed under severe zinc limitation) and the low-affinity Zrt2 (expressed under mild zinc limitation) plasma membrane zinc transporters (Zhao and Eide 1996). In contrast, *C. albicans* zinc assimilation machinery includes pH-dependent plasma membrane zinc transporters: Zrt2 (required under acidic pH) and Zrt1 (required under alkaline pH) (Crawford, Lehtovirta-Morley et al. 2018). Such a system is similar to that found in *A. fumigatus* (Amich, Vicentefranqueira et al. 2010). A *C. albicans zrt2*Δ/Δ mutant has a severe growth defect under low zinc levels in comparison to the wild type. We analyzed the transcriptional response of the wild type and *zrt2*Δ/Δ to zinc limitation and zinc re-feeding conditions. The absence of Zrt2 leads to even stronger oxidative stress response than in the wild type, signal transduction and an unfolded protein response disturbance, indicating the particular importance of Zrt2 even in the presence of Zrt1 under low zinc levels (manuscript in preparation Skrahina, Wilson et al.).

Host niches dramatically differ in zinc content (Plum, Rink et al. 2010) and thus *C. albicans* has to cope with severe zinc fluctuations. Csr1 is the major regulator of zinc homeostasis genes and is highly conserved within fungi (Zhao and Eide 1997, Moreno, Ibrahim-Granet et al. 2007, de Oliveira Schneider, Fogaça et al. 2012). Additionally, in *C. albicans* Sut1 was implicated to positively control zinc homeostasis genes *in vivo* (Xu, Solis et al. 2015). As *C. albicans* possesses four TFs (Sef1, Hap43, Sfu1, and Rim101) to control iron homeostasis (Baek, Li et al. 2008, Chen, Pande et al. 2011) we were wondering if there are more TFs essential for regulation of zinc homeostasis genes. Therefore, we screened a transcription factor deletion library (Homann, Dea et al. 2009) and indeed identified the *ssn6*Δ/Δ mutant that possessed a severe growth defect under zinc limitation in comparison to wild type growth (manuscript in preparation Skrahina, Wilson et al.). Ssn6-Tup1 complexes are known to be transcriptional repressors in eukaryotes (Braun and Johnson 1997, Jimenez, Paroush et al. 1997, Grbavec, Lo et al. 1999). In *C. albicans*, Ssn6 mediates gene repression when in a complex with Tup1, other DNA binding proteins (DBPs), or alone (Hwang, Oh et al. 2003, Garcia-Sanchez, Mavor et al. 2005, Hernday, Lohse et al. 2016). In addition to its repression function, Ssn6 is a positive regulator of filamentous growth in *C. albicans* (Hwang, Oh et al. 2003). Our findings indicate that Ssn6 is an activator of zinc transporter genes (*ZRT1*, *ZRT2*, and *ZRT3*) under zinc deprivation and thus, is a novel regulator of zinc homeostasis (manuscript in preparation Skrahina, Wilson et al.).

The distinct fungal strategy to counteract zinc toxicity

Both the deficiency and overload of zinc are harmful for cells, and thus all organisms have evolved mechanisms to regulate intracellular zinc levels. This includes

zinc importers (Zrt-, Irt-like Protein (ZIP) in eukaryotic or ZnuACB in prokaryotic cells) and zinc exporters (cation diffusion facilitator (CDF)). ZIP and ZnuACB systems are used to transport zinc from the extracellular space into the cytoplasm and from the cytoplasm into organelles. CDF transporters work in the opposite manner, exporting zinc from organelles into the cytoplasm and from there to the outside (Nies and Silver 1995, Patzer and Hantke 1998, Guerinot 2000, Gaither and Eide 2001).

The CDF transporters play a critical role in counteracting zinc overload. Cells of multicellular eukaryotic organisms contain both organelle-to-cytoplasm and cytoplasm-to-extracellular space zinc transporters. Prokaryotes lack membrane-bound organelles and thus contain only cytoplasm-to-extracellular space zinc transporters. Fungi possess only organelle-to-cytoplasm zinc transporters (based on a homology search). This phenomenon could be explained by the fact that zinc ions excreted by cells of multicellular organisms may still stay inside the organism and thus can be further used in case of an urgent need. Due to the lack of organelles prokaryotes are not able to store zinc, and in order not to poison themselves under high zinc levels express plasma membrane zinc exporters. Fungi follow a different strategy: Under external high zinc levels fungi store zinc in their organelles. For example, *S. cerevisiae* utilizes zinc transporters Zcr1 and Cot1 to import zinc into the vacuole up to the concentration of 100 mM (MacDiarmid, Gaither et al. 2000, Simm, Lahner et al. 2007). Paradoxically, *S. cerevisiae ZRC1* is expressed even under low extracellular zinc levels. Interestingly, the presence of *ZRC1* transcript levels under conditions of zinc limitation is required for the detoxification of zinc in the case of a rapid change from low to high zinc levels in the environment (MacDiarmid, Milanick et al. 2003). Similarly to *S. cerevisiae*, Zcr1 in *Cryptococcus neoformans* is required for zinc import into vacuoles (Cho, Hu et al. 2018). Additionally, *S. cerevisiae* possess specialized endosomal structures called zincosomes that are able to accumulate zinc, however, the mechanism of zinc import and the exact nature of zincosomes in *S. cerevisiae* remains unknown (Devirgiliis, Murgia et al. 2004). Similar to *S. cerevisiae*, *C. albicans* is able to accumulate zinc in both vacuoles and zincosomes. The mechanism of zinc uptake differs from *S. cerevisiae* as Zrc1 in *C. albicans* is essential for zincosomal zinc uptake, but not for vacuolar zinc accumulation (Crawford, Lehtovirta-Morley et al. 2018). Another variant to counteract zinc toxicity was observed in the fission yeast *Schizosaccharomyces pombe,* which is able to store and detoxify zinc in the endoplasmic reticulum (ER) *via* the zinc transporter Zhf. In addition, in response to high zinc levels the *S. pombe* metallothionein Zym1 sequesters zinc; this strategy has only been described for this fungus (Borrelly, Harrison et al. 2002, Clemens, Bloss et al. 2002).

Organelles not only detoxify zinc, but also serve as zinc storage to provide zinc in times of extracellular zinc starvation. *S. cerevisiae* is able to extract zinc from the vacuoles *via* the Zrt3 vacuole zinc exporter (MacDiarmid, Gaither et al. 2000). The orthologues of *S. cerevisiae ZRT3* were also found to be upregulated in response to zinc limitation in *C. albicans*, *B. dermatitidis*, and *C. dubliniensis* (manuscript in preparation Skrahina, Wilson et al.) (Böttcher, Palige et al. 2015, Munoz, Gauthier et al. 2015), indicating a similar zinc retrieval strategy from vacuolar storages.

It is important to note that the metal storage strategy is not limited to zinc. *S. cerevisiae* is able to accumulate other essential metals such as manganese, iron, nickel, and copper in its vacuoles (Raguzzi, Lesuisse et al. 1988, Nishimura, Igarashi et al. 1998, Rees, Lee et al. 2004, Culotta, Yang et al. 2005). However, highly toxic nonessential heavy metals such as cadmium are treated differently: *S. cerevisiae* contains cadmium efflux pumps in the plasma membrane to export this toxic metal into the extracellular space (Adle, Sinani et al. 2007). The mechanism of copper distribution within *S. cerevisiae* cells is an interesting hybrid: Copper is an essential metal, but copper requirement is relatively low in comparison to other metals and it becomes highly toxic at higher concentrations. Therefore, *S. cerevisiae* is able to both store copper in the vacuoles and export it across the plasma membrane (Rees, Lee et al. 2004).

In conclusion, fungi have developed elegant machineries of zinc homeostasis. Under high zinc levels, the metal is detoxified in organelles and under low zinc levels, zinc is utilized from organelles. The ability to detoxify zinc was found to be essential for *C. albicans* and *C. neoformans* virulence (Cho, Hu et al. 2018, Crawford, Lehtovirta-Morley et al. 2018). Such a system can explain the ability of fungi to exist, survive, colonize, and thrive in various environments that differ in zinc content.

The missing link between signaling pathways and transcriptional regulation

Zap1 is a zinc sensor and zinc ions regulate both activation domains and DNA binding properties of Zap1 (Zhao and Eide 1997, Evans-Galea, Blankman et al. 2003, Qiao, Mooney et al. 2006). Zap1 is located in the nucleus independent of the extracellular and cytoplasmic zinc concentration (Evans-Galea, Blankman et al. 2003). Thus, it is unclear how Zap1, when residing in the nucleus, senses external and cytoplasmic zinc levels.

Recently, Zrt1 in *S. cerevisiae* was found to be a transceptor, which acts both as a transporter and sensor of extracellular zinc. Zrt1 signals the extracellular zinc levels *via* the PKA pathway, which plays a major role in the regulation of cell growth,

metabolism, and stress resistance (Tamaki 2007, Schothorst, Zeebroeck et al. 2017). It seems likely that *C. albicans* also possesses a zinc transceptor for similar purposes. So far, no studies on this subject were published for *C. albicans*, however, recent data suggests Zrt2 to be a transceptor protein (personal communication Duncan Wilson). The *zrt2*Δ/Δ strain has a growth defect and upregulates zinc uptake machinery genes, even in the presence of zinc, in contrast to the wild type (manuscript in preparation Skrahina, Wilson et al.). Additionally, the transcriptional data of *zrt2*Δ/Δ showed that this mutant is unable to regulate the expression of *STE50,* which codes for a protein that mediates a signal transduction *via* the regulation of MAP kinase pathway (Ramezani-Rad 2003), and cAMP/PKA and MAP kinase signalling cascades where shown to interfere (Gerits, Kostenko et al. 2008). In contrast, in the wild type strain, *STE50* possessed a zinc level-dependent pattern of transcription, providing a hint towards the link between external zinc sensing and the signal transduction (manuscript in preparation Skrahina, Wilson et al.). Furthermore, the extracellular zinc level was shown to regulate the cAMP/PKA pathway in *C. albicans.* External zinc triggers cAMP production, which leads to PKA activation, and further zinc release from the ER. The mobilization of zinc from the ER is rapid and therefore unlikely to be dependent on the transcriptional regulation *via* Zap1 (Kjellerup, Winther et al. 2018).

Thus, we hypothesize Zrt2 to be an external zinc level sensor. The zinc level-dependent activation of Zrt2 *via* phosphorylation might trigger the transport of zinc out of intracellular organelles into the cytoplasm. Thus, zinc ions might be released from the ER, zincosomes, vacuoles, and, probably, from the nucleus, leading to the rapid increase in cytoplasmic zinc levels. Zinc derived from zinc storage organelles might be used as a zinc source. Additionally, the rapid increase in the zinc level might trigger a second zinc wave event and further activation of the cAMP/PKA pathway (Tamaki 2007, Schothorst, Zeebroeck et al. 2017). Moreover, a proposed decrease in nuclear zinc levels would lead to the dissociation of zinc ions from Zap1 and thus, the activated Zap1 would cause the expression of zinc uptake genes. While still speculative, this hypothesis might explain the connection between the membrane zinc transceptor and the nuclear TF.

5.1. Conclusions

During infections the host's innate immunity employs both trace metal limitation and toxicity mechanisms in order to inhibit the growth of microbial invaders. In return, successful pathogens are able to adequately control their metal homeostasis in response to metals fluctuation. In this thesis, the nutritional immunity concept in terms of host-fungus interactions was investigated. Being an opportunistic pathogen,

C. albicans can exist as a commensal and as a pathogenic fungus and thus it inhabits various host niches that differ in metal availability. We showed that in *C. albicans* the suppression of iron assimilation processes, under elevated iron levels, requires the function of the TF Sfu1. This is different in *A. fumigatus,* where, in addition to SreA (an orthologue of Sfu1 in *C. albicans*), the presence of HapX (an orthologue of Hap43 in *C. albicans*) is essential under high iron concentrations. We demonstrated that in order to wrestle with host mediated zinc withdrawal *C. albicans* has evolved sophisticated systems, including pH regulated plasma membrane zinc transporters, and that such a zinc uptake system is similar to those in *A. fumigatus*. The transcriptome data of *C. albicans* showed that external zinc limitation affects processes such as zinc homeostasis, oxidative stress, transcription, translation, metabolism, and biosynthesis, which mirrors the response to low zinc levels in *S. cerevisiae*. The transcriptional data of *zrt2*Δ/Δ under zinc deprivation conditions give us a strong hint to believe Zrt2 to be a transceptor protein. Additionally, in order to find novel TFs that regulate zinc homeostasis, we screened a transcription factor deletion library and found Ssn6 to be required for the activation of zinc uptake machinery genes under low zinc levels. To circumvent elevated zinc levels *C. albicans* imports zinc into zincosomes *via* the Zcr1 transporter, which is a vacuolar zinc importer in *S. cerevisiae*. We discovered that both zinc uptake and detoxification systems are essential for *C. albicans* virulence.

Overall, the battle for micronutrients is a fundamental element of host–pathogen interactions and thus represents various opportunities for therapeutic potential. As seen from nutritional immunity studies in host-bacteria interactions, the most extensively characterized field, strategies such as siderophore-antibiotic conjugates and gallium quenching have already been successfully applied in clinical settings. In general, fungal diseases could also be treated through modulating or blocking their metal homeostasis systems, which could potentially result in the inhibition of fungal growth and virulence. We identified that fungal pathogens represent both common and exclusive strategies to counteract metal fluctuations. Thus, it is particularly important to gain deeper insights into the specific strategies of individual pathogenic fungal species to cope with external metals bioavailability, so that future therapies would be both highly effective and targeted.

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7. Appendix

7.1. Abbreviations

ALS	agglutinin-like sequence
ABC	ATP-binding cassette
BMP2	bone morphogenetic protein 2
cAMP	cyclic adenosine monophosphate
CDF	cation diffusion facilitator
Co	cobalt
Cr	chromium
Cu	copper
DBP	DNA binding protein
ER	endoplasmic reticulum
Fe	iron
Fe^{2+}	ferrous
Fe^{3+}	ferric
Ga	gallium
GAS	Group A *streptococcus*
HCMV	human cytomegalovirus
HFE	hemochromatosis-associated protein
HPV	human papillomavirus
HSP	heat shock protein
IFN	type I interferon
IL-6	interleukin-6
MAP	mitogen-activated protein
Mn	manganese
MT	metallothionein
NET	neutrophil extracellular trap
Ni	nickel
NRAMP1	natural resistance-associated macrophage protein 1
PBP	periplasmic binding protein
PKA	protein kinase A
RNS	reactive nitrogen species
ROS	reactive oxygen species
Sap	secreted aspartic protease
SOD	superoxide dismutase
TCA	tricarboxylic acid
TF	transcription factor
TfR1	transferrin receptor 1
V	vanadium
WHO	World Health Organization
ZIP	Zrt-, Irt-like Protein
Zn	zinc

7.2. *Curriculum vitae*

Personal profile

First name: Volha

Last name(s): Skrahina Aliaksandrovna

Date of birth: 02.01.1990 in Minsk

Nationality: Belarus

Family status: Single

Parents: Dr. Alena M. Skrahina and Dr. Aliaksandr E. Skrahin

Home address: Mühlenstraße 49, 07745, Jena, Germany

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Course of studies

09.2012 – present

PhD student. Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute (HKI). Supervisor: Prof. Bernhard Hube. Thesis: "Role of micronutrients during fungal infections"

09.2007 – 06.2012

Diploma in Biology. Belarusian State University. Lecturer in Biology and Chemistry

10.2008 – 03.2011

Undergraduate student researcher. Republican Theoretical and Practical Centre «Mother and Child». Laboratory of Cytogenetic, Molecular-Genetic, and Morphological Research. Supervisors: Dr. Konstantin A. Mosse and Dr. Tatsiana V. Osadchuk. Project: "Investigation of mutations in hereditary diseases"

07.2011 – 10.2011

Undergraduate student researcher. Max Planck Institute for Infection Biology. Immunology Department. Supervisors: Prof. Dr. Dr. h.c. Stefan H. E. Kaufmann and Dr. Joerg Schreiber. Project: "MicroRNA mediated regulation of the innate immune response in tuberculosis infection"

Publications

2017

Volha Skrahina, Matthias Brock, Bernhard Hube, Sascha Brunke. *Candida albicans* Hap43 domains are required under iron starvation but not excess. **Frontiers in Microbiology**. 2017 December 1;8:2388. doi: 10.3389/fmicb.2017.02388. eCollection 2017

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Volha Skrahina, Duncan Wilson, Bernhard Hube, Sascha Brunke. Combined transcriptomics and transcription factor functional analysis of *Candida albicans* in response to zinc limitation. In preparation for Metallomics

Posters

2013

International Leibniz Research School for Microbial and Biomolecular Interactions (ILRS) symposium. Jena, Germany. Title: “Micronutrients in fungal infections”

2015

66. Mosbach Colloquium - "Metals in Biology - Cellular Functions and Diseases". Mosbach, Germany. Title: “*Candida albicans* and zinc”

International Society for Human and Animal Mycology (ISHAM) conference. Melbourne, Australia. Title: “*Candida albicans* and zinc”

2016

Annual Conference of the Association for General and Applied Microbiology conference. Jena, Germany. Title: “*Candida albicans* and trace heavy metal”

German Society for Hygiene and Microbiology (Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)) conference. Ulm, Germany. Title: “*Candida albicans* and trace heavy metal”

2017

British Society for Medical Mycology (BSMM) conference. Birmingham, United Kingdom. Title: “*Candida albicans* and trace metals”

Appendix

Talks

2011

Tuberculosis Academy conference. Innsbruck, Austria. Title: “PCR in tuberculosis diagnostics”

2013

DGHM workshop. Hannover, Germany. Title: “Micronutrients in fungal infections”

15th INTERNATIONAL European Molecular Biology Laboratory (EMBL) symposium. Heidelberg, Germany. Title: “Micronutrients in fungal infections”

2014

Central European Summer Course on Mycology workshop. Szeged, Hungary. Title: “*Candida albicans* and trace metals”

ILRS symposium. Jena, Germany. Title: “*Candida albicans* and trace metals”

2015

Zinc – UK meeting. Cambridge, United Kingdom. Title: “*Candida albicans* and trace metals”

DGHM conference. Jena, Germany. Title: “*Candida albicans* and trace metals”

2016

ILRS joint symposium. Lutherstadt Wittenberg, Germany. Title: “*Candida albicans* and trace metals”

2017

ILRS symposium. Jena, Germany. Title: “*Candida albicans* and iron”

Additional training activities

2011

School teacher of Biology Internship. School № 138. Minsk, Belarus.

School teacher of Biology Internship. Gymnasium № 13. Minsk, Belarus.

2013

Industry Partner Internship. Wacker Biotech GmbH. Research & Development Department. Jena, Germany. Work under Good Manufacturing Practice (GMP)

2014

Microscopy Course. Max Planck Institute for Chemical Ecology. Jena, Germany.

Adobe Photoshop and Illustrator Course. HKI. Jena, Germany.

Contribution to the “Long Night of Science” and “Girls’ Day / Boys’ Day” at the HKI. Jena, Germany.

2015

ILRS workshop “Professional orientation for PhD students in the life sciences”. HKI. Jena, Germany.

Practical course "Infection Biology of Pathogenic Yeasts" supervisor for microbiology students. HKI. Jena, Germany.

2016

ILRS workshop “Leadership skills”. HKI. Jena, Germany.

Travel grants

2011

Tuberculosis Academy conference. Innsbruck, Austria.

2013

DGHM workshop. Hannover, Germany.

2015

ISHAM conference. Melbourne, Australia.

Awards

2015

66. Mosbach Colloquium - "Metals in Biology - Cellular Functions and Diseases". Mosbach, Germany. **Best Poster Award**

2016

DGHM conference. Ulm, Germany. **Best Electronic Poster Award**

2017

BSMM conference. Birmingham, United Kingdom. **Best Elevator Presentation Prize**

7.3. Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Mir ist die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt. Personen, die mich bei den Experimenten, der Datenanalyse und der Verfassung der Manuskripte unterstützt haben, sind als Ko-Autoren auf den entsprechenden Manuskripten verzeichnet. Personen die mich bei der Verfassung der Dissertation unterstützt haben, sind in der Danksagung der Dissertation vermerkt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch bei keiner anderen Hochschule als Dissertation eingereicht und auch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung verwendet.

Jena, den 12.04.2018

_____

Volha Skrahina

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My sincere thanks go to the deputy head of the MPM department, who was also my second supervisor, Dr. Sascha Brunke, for the splendid guidance of my thesis. Special thanks for his constructive criticism and great patience, as he was able to solve plenty of my problems, including laboratory experiments, information technology (IT) support, and scientific issues.

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